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THYROIDAL UTILIZATION OF RADIOIODINE IN THE WHITE-THROATED SPARROW AND WEAVER FINCH[1]

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ABSTRACT

The fate of radioiodine injected into white-throated sparrows (*Zonotrichia albicollis*) collected in November, or weaver finches (*Euplectes afer taha*) collected in February or May, was followed. The thyroid of the white-throated sparrows accumulated an average maximum of about 60% of the injected dose within 6 hours. It was unusual in the high proportion of monoiodotyrosine formed.

The February weaver finches concentrated 73% of the injected I^{131} in their thyroids, the May finches 57%. Additional dietary iodine did not significantly affect the thyroidal uptake. Thyroxine given to *Euplectes* did not appreciably affect the distribution of I^{131} in the different chemical fractions in the thyroid, but reduced uptake almost 100-fold from 57% to a maximum of 0.59%.

Both species formed thyroxine as well as triiodothyronine. The proportion of administered radioiodine found as thyroxine in the thyroid of the weaver finch was unusually small, 10.7% in February and 2.4% in May, although in white-throated sparrows it was about 24.3%.

THE thyroid gland of wild birds is of considerable theoretical interest because of its seasonal variations in structure, and its inferred relation to such phenomena as migration and reproduction (1). Despite this general interest little direct research has been concerned with the endocrine activity of the avian thyroid gland. The few studies which have been made in this field have utilized almost exclusively the recently hatched chick (2, 3, 4).

In this connection we have investigated the utilization of radioiodine in the thyroid glands of two species, the white-throated sparrow, *Zonotrichia albicollis*, and the South African weaver finch, *Euplectes afer taha* (A.

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Smith). The white-throated sparrow is a migratory species and was tested in the autumn, shortly before migration. The weaver finch is a non-migratory form.

MATERIALS AND METHODS

Male and female white-throated sparrows were trapped on the campus of Northwestern University at the end of September and tested five weeks later. They were caged in a room maintained at 20°–25° C and subjected to uniform 12-hour light periods throughout the experiment. Water was given freely, as was canary seed containing 0.7 μg. of iodine per 100 gm. of food.[3] The light sources were two 40 watt fluorescent bulbs, placed about 90 cm. from each bird cage. Each cage (60 cm. long by 48 cm. high by 35 cm. deep) contained five to six birds. Fourteen μc. of I^{131} as carrier-free iodide in 0.1 cc. of saline solution were injected by the intraperitoneal route. After I^{131}-injection the birds were killed at 6, 12, 24, 48, 72, and 120 hour intervals, and their thyroids were removed and used for the radioactivity measurement and chromatography.

The weaver finches were purchased from a commercial source and trans-shipped by air from Holland about one week after capture in South Africa. Two series of experiments were carried out with this species, one in February, and the second in May. In the February experiments, twenty-eight male and female finches were kept for two weeks at a constant temperature and constant daily photoperiod (22° C, 12 hours illumination per day). Light came from a 100 watt electric bulb placed 1.5 m. from the bird cages. The birds were divided among 4 cages (63 cm. long by 30 cm. high by 30 cm. deep), each containing 7 birds. The food was canary seed containing 1.1 μg. of iodine per 100 gm. of food. After two weeks under these conditions, 15 μc. of radioiodine in 0.1 cc. of saline were injected intraperitoneally, and the birds were killed at various time intervals thereafter.

Male and female weaver finches used in the May experiment were kept under the same conditions except for the diets fed. From the day the birds were received in the laboratory (May 13), their diets consisted of canary seed, or canary seed supplemented with KI. The iodine contents of the seed, and the iodine-enriched seed were 3.3 μg. and 13.0 μg. of iodine in 100 gm. of food respectively. On May 15, half of the finches fed normal food were implanted intraperitoneally with thyroxine pellets weighing 4.2 to 6.7 mg. The pellet contained carbowax (25%), lactose (25%), and DL-thyroxine (50%). All birds were injected with 15 μc. of I^{131} in 0.1 cc. of saline solution 5 days after beginning the experiment (May 18). They were sacrificed at intervals thereafter up to 120 hours.

Thyroid glands were individually homogenized in 0.9% saline solution. An aliquot of the solution was taken for a measure of total thyroidal radioactivity in a scintillation counter. An aliquot of the originally injected solution was counted under similar conditions each time and the thyroidal uptake was expressed as the percentage of the administered isotope found in the organ. Chromatographic analysis of the chemical forms of the thyroidal iodine was done by a technique closely following that of Lissitzky (5). Briefly, the homogenate was digested at 37° C for 48 hours with trypsin at pH 8.5, followed by 24 hours of papain at pH 5.3. The hydrolyzed mixture was then extracted with acidified butanol and the extract was evaporated to dryness at temperatures maintained below 45° C. The concentrated extractable iodine compounds were applied to filter paper for descending chromatography by butanol-acetic acid (BAC) and butanol-ammonia (BAM) solvents. For identification of radioactive spots, known potassium iodide (KI), monoiodotyrosine (MIT), diiodotyrosine (DIT), triiodothyronine (TIT[3]),

[3] Analysis of the iodine content of the food was made by the Albert R. Chaney Laboratory, Glendale, California.

and thyroxine (Tx) were chromatogrammed in parallel with the hydrolyzed thyroidal material. The distribution of radioactivity along the length of the filter paper chromatograms was determined, usually with an end-window Geiger tube and recorded automatically on graphs. The total radioactivity of chromatograms was expressed in terms of the numbers of squares of the graph covered by the radioactivity curve. The number of squares making up the activity curves of iodide or radioiodinated amino acids was counted separately, and the percentage of total extractable radioactivity of each of these compounds was calculated.

RESULTS

Uptake of I^{131} by thyroid gland

The accumulation of I^{131} in the white-throated sparrow's thyroid was very rapid, rising within 6 hours to a maximum of about 60% of the injected dose. The peak quantity of I^{131} was held by the gland until 48 hours after the injection. At 72 and 120 hours the thyroid held about 57% and 30% of its peak accumulation, respectively (Fig. 1).

In the first (February) series of weaver finches, the maximum uptake similarly was achieved by 6 hours after I^{131}-injection, and it reached a

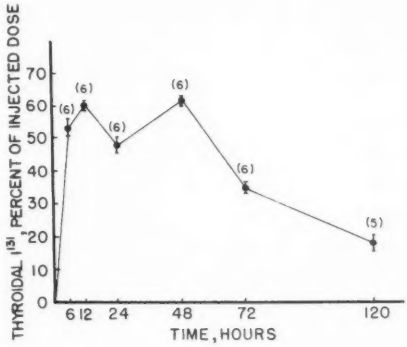

FIG. 1. Uptake of I^{131} by thyroid gland of white-throated sparrows as percentage of injected dose. The vertical lines show standard errors, and the figures in parentheses represent number of birds.

value about 70% of the injected dose (Fig. 2). There followed a plateau of thyroidal radioiodine until 48 hours after I^{131}-injection. The radioiodine content of the thyroid gland decreased at a rate and in a pattern resembling that in the white-throated sparrow. At 72 and 120 hours the value was about 80% and 35% of the peak accumulation, respectively. In the May finches (Table 1, Fig. 2), the maximum uptake in normal birds (57.3%) was lower than that (70%) of the birds tested in February. The maximum uptake was found at 6 hours after I^{131}-injection and, as before, the thyroidal radioiodine decreased gradually after 48 hours. The pattern of isotope uptake and loss was similar in the two experiments. In the birds fed KI-diet, the maximum uptake (54.7%) was delayed to about 30 hours after I^{131}-injection, significantly later than in birds fed a normal diet (Table 1). However, the difference in maximum uptake between normal birds and

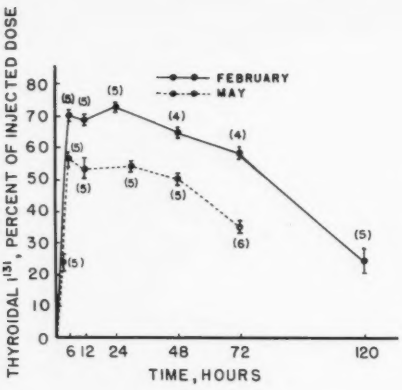

FIG. 2. Uptake of I^{131} by thyroid gland of weaver finches as percentage of injected dose. The vertical lines show standard errors, and the figures in parentheses represent number of birds.

KI-birds was not statistically significant. The uptake of I^{131} was remarkably inhibited in the birds implanted with thyroxine pellets. The maximum uptake, about 0.6%, occurred 6 hours after I^{131}-injection (Table 1).

The thyroid weight of the birds implanted with thyroxine pellets decreased significantly within the 3 days after the implantation and continued to decrease in the birds killed 5 and 6 days after implantation. The birds fed the KI-diet showed no change in thyroid weight (Table 2).

TABLE 1. RADIOIODINE ACCUMULATION (EXPRESSED AS PERCENTAGE OF INJECTED DOSE) AND RELATIVE PROPORTIONS OF RADIOIODINATED AMINO ACIDS IN THYROIDS OF MAY EXPERIMENTAL WEAVER FINCHES (EXPRESSED AS PERCENTAGE OF TOTAL EXTRACTABLE RADIOACTIVITY)

Treatment	Hours after I^{131} injection	Thyroid uptake, % of total injected dose	(MIT, DIT)	MIT/DIT	I^-	Tx	TITN	Unk. A	Unk. B
Control	3	24.3 ± 5.8 (5)†	71.0	1.3	5.6	0.8		7.5	0.8
	6	57.3 ± 5.1 (5)	70.9	1.2	4.7	1.2		4.6	0.4
	12	53.2 ± 5.9 (5)	73.5	1.2	7.4	0.9			1.2
	30	54.7 ± 3.8 (5)	74.8	0.7	3.3	0.7		1.7	0.3
	48	50.5 ± 3.5 (5)	77.0	0.5	3.8	2.4			
	72	35.3 ± 4.0 (6)	74.0	0.6	5.7	1.3			1.3
Birds implanted with thyroxine pellet*	3	0.29 ± 0.06 (5)	72.5	1.1	8.6	0.5	0.1	1.3	1.1
	6	0.59 ± 0.12 (3)	82.7	1.0	4.8	1.8		7.9	0.9
	12	0.51 ± 0.12 (4)	87.3	1.4	4.8	0.6		0.3	1.0
	48	0.25 ± 0.10 (3)	83.6	0.8	4.0	1.2		0.2	0.7
	72	0.57 ± 0.14 (3)	76.1	0.6	4.0	2.0		1.3	
Birds fed with KI-diet	3	26.8 ± 2.9 (5)	73.3	1.4	7.3	1.0			1.0
	6	28.8 ± 5.0 (4)	83.0	0.6	3.5	1.5			0.2
	12	46.9 ± 5.5 (4)	60.5	1.0	3.9	1.1		1.2	0.8
	30	54.7 ± 4.2 (5)	47.0	0.5	—	1.1		1.6	2.1
	48	41.5 ± 5.7 (5)	77.3	0.6	4.0	3.0	0.7	2.1	0.4
	72	45.0 ± 3.2 (5)	76.7	0.3	3.1	2.3		2.1	0.6

* Thyroids of birds killed 30 hours after I^{131} injection were not studied.
† Figures in parentheses indicate numbers of birds in each test.

TABLE 2. MEAN THYROID WEIGHTS, WITH STANDARD ERRORS, OF WEAVER FINCHES FED KI-ENRICHED DIET, OR IMPLANTED WITH THYROXINE PELLETS (MG)

Treatment	3 days after treatment	5 days after treatment	6 days after treatment	7 days after treatment	8 days after treatment
Birds fed KI-diet		1.5±0.04 (13)*		1.8±0.15 (10)†	1.4±0.20 (5)‡
Birds implanted with thyroxine pellet and fed normal diet	1.3±0.07 (13)*	1.0±0.23 (3)†	1.0±0.12 (3)‡		
Controls	1.6±0.05 (14)*,	1.7±0.02 (10)†,	1.6±0.16(6)‡		

* The data from birds killed at 3, 6 and 12 hours after radioiodine injection were pooled and recorded in this column. Figures in parentheses show number of birds.
† The data from birds killed at 30 and 48 hours after radioiodine injection were pooled and recorded in this column.
‡ The data from birds killed at 72 hours after radioiodine injection were pooled and recorded in this column.

Radiochromatography

White-throated sparrow. At any interval from 6 to 120 hours after injecting the labelled iodine the largest part of it was found in the forms of MIT and DIT (Table 3). The ratio of radioactive MIT to radioactive DIT was always greater than 1. Tx and TITN appeared in the proportions of 4.3 and 2.1 per cent respectively 6 hours after I^{131}-injection. Their total, 6.4%, increased to a maximum of 27.6% at 72 hours, but at 120 hours decreased to 14.6%. The proportion of TITN remained quite uniform and low (2.1–3.3%). Two unknown substances of uniform Rf value, A and B, were detected consistently in chromatograms. Their maximum proportions of 14.4% (A) and 4.4% (B) appeared 48 hours after I^{131}-injection. The Rf of A was approximately 0.56 in BAM, and that of B was around 0.10 in BAC.

Weaver finch, February experiment. The results are summarized in Table 4. Most of the radioiodine in thyroid glands of February weaver finches remained in the fractions of MIT and DIT at any interval tested. DIT always exceeded MIT, contrary to the white-throated sparrow. The ratio MIT/DIT showed no measurable change throughout the period of study. As in the white-throated sparrow, the proportion of labelled TITN remained small and uniform; radiothyroxine increased from 6 hours to 48 hours (7.8% to 10.7%), but at 120 hours it decreased to 3.8%. No radioactive TITN was detectable at this time. Thus, despite a higher maximum thyroidal I^{131}-uptake in the thyroid gland of the February weaver finch, a smaller proportion of the I^{131} was found as hormone. Two unknown sub-

TABLE 3. RELATIVE PROPORTIONS OF RADIOIODINATED AMINO ACIDS IN THYROID OF WHITE-THROATED SPARROWS, EXPRESSED AS PERCENTAGE OF TOTAL EXTRACTABLE RADIOACTIVITY

Hours after I^{131} injection	(MIT, DIT)	MIT/DIT	I^-	Tx	TITN	Unk. A	Unk. B
6	77.5	2.3	1.0	4.3	2.1	4.4	1.5
12	58.4	1.2	4.4	(21.3)*		9.3	1.9
24	63.8	2.6	2.1	16.5	2.1	6.0	2.1
48	45.0	1.5	4.3	(22.8)*		14.4	4.4
72	56.4	1.8	3.6	24.3	3.3	8.4	1.8
120	67.2	2.0	3.6	(14.7)*		10.0	1.8

* Tx and TITN were not separated in chromatogram.

TABLE 4. RELATIVE PROPORTIONS OF RADIOIODINATED AMINO ACIDS IN THYROID OF FEBRUARY WEAVER FINCHES, EXPRESSED AS PERCENTAGE OF TOTAL EXTRACTABLE RADIOACTIVITY

Hours after I^{131} injection	(MIT, DIT)	MIT/DIT	I^-	Tx	TITN	Unk. A	Unk. B
6	62.7	0.6	2.8	7.8	1.4	10.4	10.9
12	59.1	0.7	1.9	8.9	1.3	13.1	5.1
24	65.5	0.7	1.7	7.4	0.5	14.4	4.2
48	56.6	0.7	1.5	10.7	1.1	25.4	9.1
120	62.2	0.7		3.8		12.5	15.1

stances were detected also in this bird. The Rf of A (10–25%) was about 0.36 in BAM and that of B (4–15%) was 0.12 in BAC.

Weaver finch, May experiment. As in all other chromatographic analyses, the sum of MIT and DIT constituted the largest fraction of the radioactive label in the thyroid. In contrast with the February weaver finches, the percentage of I^{131} in MIT exceeded that of DIT at first, but after about 30 hours following I^{131}-injection, the situation became reversed (Table 1). In February weaver finches radioactive DIT exceeded MIT at all times tested. The most unusual observation in these birds was that labelled Tx remained lower in percentage (0.7–3.0%) throughout the experiment than in any of the other experiments and TITN could be detected only in traces. The two unknown substances (A and B) were similarly very small in percentage, and sometimes they were not detectable.

DISCUSSION

As studies of the utilization of radioactive iodine in different vertebrate groups have accumulated, it has become possible to recognize patterns of thyroid function, some of them characteristic of particular animal groups (6, 7). In mammals, in general there is a rapid accumulation of isotope and then a discharge phase is associated with release of labelled hormone. In some teleostean fishes a similar pattern is found, but in others, only a minute fraction of the available radioiodine is accumulated by the thyroid, a very slow rate of turnover is apparent, and little or no newly labelled hormone can be detected. Such inactive thyroids have been found in most of the amphibians and reptiles tested. In reptiles, and perhaps also in amphibians (8, 9) the phase of thyroidal radioiodine accumulation may be extremely prolonged due to continued, though slow, transfer from some tissue site of iodine "storage" to the thyroid gland.

In birds almost all of the limited research that has been done has utilized young chicks. Fink (10) has tested thyroidal radioactivity of two I^{131}-injected captive wild species, white-crowned sparrows (*Zonotrichia leucophrys pugetensis* and *Zonotrichia leucophrys gambelii*) and brown towhees (*Pipilo fuscus*). From this work, unfortunately, the only conclusion drawn was that the peak of I^{131}-uptake is reached 15 to 17 hours after injection. By

ontrast, in the species tested by us, the white-throated sparrow and eaver finch, this peak was reached sooner, by about 6 hours after admin- tration of the isotopic iodine. In the starved chick, according to Bates and ornfield (11), this peak is reached before 16 hours; in the fed 7–10 week ockerel (2), it is reached between 2 and 8 hours.

The fraction of the administered dose of I^{131} accumulated by the thyroid quite variable from one species to the next and thus forms part of no rec- gnizable pattern. In fact, one of the puzzling aspects to those who have sted this property in newly hatched chicks is its great variability from e week to the next, and with season. Bates and Cornfield (11) have pub- hed a range of 20% to 60% as having been found by them over a period time. We have found mean thyroidal uptakes, particularly in summer, of s than 1.0% in White Leghorn chicks (12). However, these variations in chick thyroidal I^{131}-uptake are quite capricious and unpredictable, and season has only a broadly recognizable influence. In a bird as genetically and presumably physiologically uniform as the White Leghorn chick, this is difficult to understand. The data from the wild species we have tested are comparatively much less variable, and the thyroidal uptake surpris- ingly high.

The high iodine uptake found by us in the white-throated sparrow and weaver finch may be due, at least in part, to the low iodine content of the diet of canary seed fed during the period of the experiment. However, since increasing the dietary iodine by a factor of four had no appreciable effect on thyroidal I^{131}-uptake (Table 1) in the finches, other factors may make nec- essary a more complex interpretation. Frey and Albert (3) state that both starved chicks and chicks fed a commercial mash have the same thyroidal I^{131}-uptake. Accordingly, it must be concluded that the chick's thyroidal radioiodine uptake and content, as well as that of the finches and sparrows, is not sensitive to the fluctuations in dietary iodine represented in the re- spective experiments. The basis for this unresponsiveness to dietary iodine is not readily apparent and will require further study. On the other hand, peak thyroid uptake of tracer iodine is extremely sensitive to parenteral thyroxine, being reduced (Table 1) about 100-fold in the weaver finch ex- periment, by an implanted carbowax-thyroxine pellet. This responsiveness suggests that the control of TSH secretion is readily influenced by thy- roxine, and that thyroid function can vary between very wide limits within this sphere of control.

As remarkable as the rapid and strong thyroidal accumulation of radio- iodine is the length of time this high concentration is maintained in the gland. Two days after the strong accumulation phase, the thyroids still contained almost the peak quantity of the I^{131} accumulated. In four days only about half of the original radioactivity level had been lost. Such pro- longed maintenance of a high I^{131} concentration could be due to either, or both, a slow rate of hormonal release, or a rapid peripheral deiodination of

secreted hormone and rapid thyroidal reaccumulation of the freed iodine. Since this study was limited to the intrathyroidal phase of metabolism of radioiodine in the bird, it is not possible to say to what extent hormonal "turnover" occurred, or to otherwise explain the long "retention" of tracer iodine in a thyroid which is as apparently active as the bird's. Similar apparent retention of radioiodine has been described for all birds appropriately studied so far (Table 5) with the possible exception of 7–10 week old cockerels (2), so that this may be the most characteristic feature of thyroidal function in birds. However, further work will be required to show whether this is indeed an "avian pattern" of thyroid function.

TABLE 5. SUMMARY OF PATTERNS OF UTILIZATION OF RADIOIODINE BY THYROIDS OF VARIOUS SPECIES OF BIRDS

Species	Peak thyroidal uptake, per cent of dose	Time of peak uptake, hours after injection	Per cent of peak uptake remaining in thyroid			Diet fed	Authority
			48 hr.	72 hr.	96 hr.		
Domestic fowl, day-old chicks	20–60 depending on season	16	89*	90*	82*	Starved	Bates and Cornfield, 1957
Domestic fowl, ½–2 day old chicks		8	86† 91‡	83† 46‡		Commercial feed "Punaheltta"	Wahlberg, 1955
Domestic fowl, 7–10 week old cockerels	10–17	2–8	40–55*	45*	45*	Wheatmeal, oatmeal, barleymeal mixture	Vlijm, 1958
White-crowned sparrow, and brown towhee adults		15–17	80–90*	60–80*	50–65*	Canary seed	Fink, 1957
White-throated sparrow adults	61	6–12	100	57	44*	Canary seed	(this report)
Weaver finch adults	73 (Feb.) 57 (May)	6 6	89 88	80 61	56*	Canary seed	(this report)

* Figure not given directly by author, but calculated from data presented.
† Experiment with carrier-free I^{131}.
‡ Experiment using I^{131} with carrier.

An unusual finding in the chromatographic analyses is the high proportion of radioactive monoiodotyrosine found in the thyroid of the white-throated sparrow (Table 3). Throughout the five days of study, I^{131} in MIT exceeded I^{131} in DIT. In the May weaver finches, on the other hand, an early excess of radioactive MIT was later (after 12 hours) exceeded by DIT (Table 1). This resembles the experience of Vlijm (2) with the cockerel. Taurog, Tong, and Chaikoff (13) on the basis of a study of rats and adult hens, concluded that the bird's thyroid contains more MIT than the mammal's. Vlijm (2) disagrees with this point of view. It is of further interest that in weaver finches whose diet was supplemented with additional iodine, the excess of DIT* over MIT* was greater than in birds on an unsupplemented canary seed diet (Table 1). This would seem to reinforce the suggestion by Leloup and Lachiver (14) that the proportion of MIT/DIT in the thyroid is influenced by the dietary iodine supply. A deficiency of dietary iodine would favor MIT production, and, eventually, triiodothyronine.

The proportion of labelled thyroxine formed by the white-throated sparrow's thyroid (peak value, 24% of total thyroidal I^{131}) seems fairly representative of other homoiotherm vertebrate thyroids (2). However, the proportion of radio-triiodothyronine seems unusually high. In this sense, the low proportion of labelled thyroid hormone in the thyroid of the weaver finch was surprising in view of the extraordinarily high avidity of the gland for iodine. It is possible, though perhaps improbable, that the thyroid hormone is released almost as rapidly as it is formed. The high proportion of "immature" forms of the hormone, MIT and DIT, in the hydrolyzed thyroprotein make this explanation appear unlikely. In the February weaver finches, whose total thyroidal radioiodine uptake averaged 73% of the total available to the entire bird, only 10.7% of this isotope (at 48 hours) was in the form of radiothyroxine. In the May weaver finches, whose total thyroid I^{131}-uptake was lower, 57%, the proportion of radiothyroxine was minute, being at most 2.4% of the accumulated radioiodine. Treatment of the May finches with thyroxine pellets, or additional dietary iodine had negligible effect on this. We believe that this combination of great avidity of the weaver finch's thyroid for iodine combined with its apparently low production of hormone is the most unusual functional property suggested by this series of experiments.

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THE ACTION OF CORTISONE ON OXIDATIVE AND GLYCOLYTIC LIVER ENZYME ACTIVITIES IN RATS OF DIFFERENT AGE AND SEX

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ABSTRACT

The activities of three mitochondrial respiratory enzymes were determined in the liver of 33 normal and 38 cortisone-treated rats of different age and sex. In addition, glucose-6-phosphate isomerase, aldolase, glycerophosphate kinase, and lactic dehydrogenase activities were determined in mitochondrial supernatants.

The activity of cytochrome oxidase in mitochondria of cortisone-treated rats was decreased, especially in rats of more than one year of age. The DPN-cytochrome c reductase activity was somewhat decreased in old cortisone-treated rats, the succinic dehydrogenase activity was unaltered in all groups. No cortisone-induced changes in the activities of the glycolytic enzymes were found.

IT HAS been shown that cortisone affects the activity in the liver of some enzymes closely related to the utilization of glucose. The activity of glucose-6-phosphatase, fructose-1, 6-diphosphatase, aldolase and glutamic-pyruvic transaminase (1–4) is enhanced, while the phosphorylase activation by muscle adenylic acid is inhibited by cortisone administration (5).

Cortisone lowers the oxygen uptake of rat liver mitochondrial preparations (6). No effect of cortisone on the oxidative phosphorylation was observed, however. An extensive study (7) on the action of cortisone on the oxidative phosphorylation in rats of different age and sex is described in the present number of this journal. Five different acids of the tricarboxylic cycle were used in the study. It was observed that the oxidative phosphorylation was uncoupled by cortisone in all the groups studied. The largest effect was observed on the utilization of phosphate. The utilization of oxygen was less inhibited. The uncoupling of the oxidative phosphorylation was most marked in old female rats when isocitric acid was used as substrate. The findings indicate that the mitochondrial function is directly influenced by cortisone. In the present investigation the effect of cortisone on the mitochondrial respiratory enzymes was taken under closer study. The changes in the activity of four glycolytic enzymes in liver were also followed.

MATERIAL AND METHODS

The samples of rat liver mitochondrial suspensions were taken from the preparations made for the simultaneous studies on the effect of cortisone on oxygen and phosphate

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TABLE 1. ASSAY MIXTURES IN THE MITOCHONDRIAL ENZYME DETERMINATIONS

	Cytochrome oxidase	DPNH-cytochrome reductase	Succinic dehydrogenase
0.3 M phosphate buffer pH 7.4	0.5	0.5	0.5
Water	2.02	1.35	1.45
Cytochrome c, 1% aqueous[1,2]	0.4	0.4	0.4
$4 \cdot 10^{-3}$ M Al_2Cl_3	0.03		
0.3 M sodium succinate			0.2
$1.2 \cdot 10^{-2}$ M DPNH[1]		0.1	
Mitochondrial suspension	0.05	0.05	0.05

[1] Product of Sigma Chemical Company, St. Louis, Missouri.
[2] Reduced with sodium hydrosulphite and aerated just before use for the cytochrome oxidase assays.

utilization and P/O ratio (7). The details and the separation of the mitochondria are described in the text.

The cytochrome oxidase (8), succinic dehydrogenase (9) and DPN-cytochrome c reductase (10) activities were measured in a Model B Beckman spectrophotometer by following the change in optical density at 550 mu at intervals of 10 seconds (Table 1). An addition of Al_2Cl_3 or $CaCl_2$ did not enhance the succinic dehydrogenase activity and was therefore omitted from the reaction mixture.

Aldolase (11), phosphoglycerate kinase (12) and lactic dehydrogenase (13) in mitochondrial supernatants were assayed by common enzymic methods. The fructose-6-phosphate formed in the glucose-6-phosphate isomerase assays in mitochondrial supernatants was measured colorimetrically (14).

RESULTS

Table 2 summarizes the experiments on the mitochondrial enzymes. The cytochrome oxidase activity tends to be low in all groups of cortisone-

TABLE 2. ACTIVITY OF THREE RESPIRATORY ENZYMES IN LIVER MITOCHONDRIAL SUSPENSIONS. CHANGE IN OPTICAL DENSITY PER MINUTE. FIGURES PLOTTED PER 10 MG. OF MITOCHONDRIAL NITROGEN

Sex	Age in months	Control group			Cortisone group		
		Number of assays	Mean	Standard deviation	Number of assays	Mean	Standard deviation
			Cytochrome C oxidase				
Male	2–3	8	0.270 ±	0.102	10	0.192 ±	0.096
Female	2–3	5	0.258	0.032	5	0.195	0.045
Female	6–8	6	0.280	0.050	5	0.291	0.049
Male	12	8	0.278	0.091	10	0.129	0.047
Female	12	6	0.304	0.074	8	0.139	0.058
			DPN-Cytochrome C reductase				
Male	2–3	6	0.168	0.063	10	0.139	0.034
Female	2–3	6	0.239	0.020	3	0.340	0.107
Female	6–8	4	0.171	0.027	5	0.209	0.032
Male	12	8	0.233	0.049	10	0.181	0.049
Female	12	6	0.241	0.063	7	0.191	0.037
			Succinic dehydrogenase				
Male	2–3	7	0.108	0.032	9	0.092	0.034
Female	2–3	6	0.124	0.022	4	0.142	0.041
Female	6–8	4	0.088	0.034	5	0.137	0.021
Male	12	6	0.089	0.046	6	0.078	0.041
Female	12	9	0.100	0.037	9	0.098	0.049

TABLE 3. ACTIVITY OF FOUR GLYCOLYTIC ENZYMES IN LIVER MITOCHONDRIAL SUPERNATANTS. ADDITION OF SUPERNATANT EQUIVALENT TO 40 MG. OF LIVER TISSUE

	Control group			Cortisone group		
	Number of assays	Mean	Standard deviation	Number of assays	Mean	Standard deviation
Glucose-6-phosphate isomerase mg. of fructose-6-phosphate formed in 10 minutes	26	0.269	± 0.104	35	0.290	± 0.118
Aldolase Change in OD at 340 mμ per minute	26	0.025	0.007	32	0.028	0.010
Glycerophosphate kinase Change in OD at 340 mμ per minute	8	0.030	0.004	15	0.032	0.018
Lactic dehydrogenase Change in OD at 340 mμ per minute	23	0.274	0.092	35	0.306	0.125

treated rats. The most marked decrease in activity was found in the groups of old rats, where the activity was a mere one-third of the control level. The least difference was noticed in the group of middle-aged rats.

The activity of DPN-cytochrome c reductase was unaltered in most groups. A decrease in activity was observed in the groups of cortisone-treated old rats. The decrease was less pronounced, however, than the reduction in the cytochrome oxidase activity in the same groups of rats.

The succinic dehydrogenase activity was unaltered in all groups of test animals.

No change in the activity of the four enzymes of the glycolytic system was found in rats treated during 7 days with 5 mg. of cortisone. The number of assays in the different groups was too small for definite conclusions, however, the over-all results of the assays are summarized in Table 3.

DISCUSSION

The inhibition of the oxygen uptake of the mitochondria by cortisone treatment (6, 7) indicates that cortisone may induce changes in the mitochondrial respiratory chain. The effect, according to the present study, seems to be located at the cytochrome oxidase level, since only minor or no changes were found in the succinic dehydrogenase and DPN-cytochrome c reductase activities. Furthermore, the effect can not be due to any general alteration in the mitochondria which would enable them to escape separation, since only negligible cytochrome oxidase activity was found in the supernatant fractions. The inhibition of the mitochondrial oxygen uptake was most marked in the old female rats in the studies of Kerppola (7). The decrease in the cytochrome oxidase activity in the present study was similarly most pronounced in old animals. Thus, the decrease in the utilization of oxygen by the cortisone-treated rats may be the result of the reduction in the cytochrome oxidase activity.

Cortisone was shown further to uncouple oxidative phosphorylation in rat liver mitochondria (7). The effect was also most marked in old female rats. This action of cortisone has not been exactly located. The finding of Kerppola (7) that the phosphorylation process usually was more disturbed than the utilization of oxygen, and the observation now made that the activity of three respiratory enzymes was only partially inhibited under cortisone treatment suggest that the action may be located somewhere in the phosphorylation process. The decrease in oxidative phosphorylation and the reduction in the cytochrome oxidase activity may thus present two different effects of cortisone.

The negligible change in the activities of the glycolytic enzymes in the present study suggests that the production of energy-rich phosphate by glycolysis is not compensatorily increased during one week of cortisone administration.

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THE EFFECT OF ESTRONE AND PARATHYROID EXTRACT ON BONE CITRATE METABOLISM

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ABSTRACT

This study was designed to further elucidate an estrone-parathyroid extract antagonism in bone. The incorporation of acetate-1-C^{14} into bone citrate was measured in the presence of both substances administered singly and in combination. Estrone increased bone citrate synthesis and concentration while parathyroid extract enhanced labeled citrate formation with little change in bone citrate concentration.

When both materials were administered the results were essentially those observed for estrone alone although the changes were not as great.

A RECENT report (1) from this laboratory described the opposing actions of estrone and parathyroid extract that became evident in studies on bone accretion in mice. It was observed that parathyroid extract reduced the effectiveness of the estrogen in enhancing Ca^{45} deposition in the tibia, and evaluation of dose-response curves indicated that the interaction of the two substances probably involved one of the systems concerned with mineral accretion in bone. The recent demonstration by Martin *et al.* (2) that parathyroid extract stimulated citrate production in bone, and the observation by Lehman *et al.* (3) that bone citrate in the pigeon increased 5-fold at the onset of the egg laying cycle, have indicated that bone citrate metabolism may be profoundly modified by either estrogenic or parathyroid hormones. It seemed reasonable, therefore, to attempt to elucidate the estrone-parathyroid antagonism by measuring the incorporation of labeled acetate into citrate in the presence of the hormones alone and in combination. This report describes such an experiment.

METHODS

Four groups of 6 adult male mice each were injected subcutaneously daily with the following materials: (1) control, corn oil alone; (2) estrone, 2 mg. per kg. in corn oil; (3) parathyroid extract (Lilly) 230 units/kg.; and (4) estrone, 2 mg. per kg. and parathyroid extract 230 units/kg. After 10 days of treatment all animals were given 5 microcuries of sodium acetate-1-C^{14} intraperitoneally and placed in metabolism cages. The expired CO_2 of each group was collected for 90 minutes, plasma samples were taken, the animals sacrificed and the left tibia and femur of each were removed for analysis.

For the separation of citric acid the chromatographic method of Kinnory *et al.* (4) was used. The bones were briefly autoclaved to facilitate cleaning, dried, weighed and dissolved in 0.5 ml. of 5N HCl with the aid of gentle heat. The tibia and femur of each

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TABLE 1. SOLVENT SERIES FOR RECOVERY OF CITRATE IN BONE DIGESTS

Fraction	Solvent	Volume (ml.)
1	Benzene	20
2	15% ethyl ether in benzene	20
3	50% ethyl ether in benzene	20
4	85% ethyl ether in benzene	20
5	100% ethyl ether	20
6	2% ethanol in ethyl ether	80

animal were treated as a single sample. Bone digests were adjusted to pH 1.4 using 3N NaOH and diluted to a volume of 5 ml. with 0.05N HCl. Aliquots (1 ml.) of the digests were mixed into 2.0 gm. portions of silicic acid and when the adsorbent appeared dry a small portion of chloroform was added to it and the slurry transferred quantitatively into a chromatography column. The silicic acid column (10×60 mm.) was packed by running 50 ml. of chloroform through it and the top was protected from disturbance by solvent addition with a disc of filter paper.

Instead of the gradient elution technic described by Kinnory a series of 6 solvent mixtures were used to elute the carboxylic acids of the digest (Table 1). Citric acid was found to be eluted quantitatively in fractions 5 and 6 and was not contaminated with the other organic acids removed in the earlier fractions. Fractions 5 and 6 were pooled, made slightly alkaline with 0.3N NaOH and taken to dryness with gentle heat. The sodium citrate was transferred quantitatively to stainless steel cupped planchets using 50% ethanol. After evaporation of the solvent the radioactivity was measured with an end window geiger tube. The citrate was then transferred quantitatively to volumetric flasks and aliquots were taken for colorimetric determination of citrate using the method of Ettinger *et al.* (5). The expired $C^{14}O_2$ was collected in absorption towers containing 2N NaOH. CO_2 was measured titrimetrically and the C^{14} from $BaCO_3$ mounts. All radioactivity measurements were corrected for self absorption. Plasma citrate was estimated by the method of Ettinger *et al.* (5).

RESULTS

Plasma, bone and CO_2 analytical data are summarized in Table 2. Estrone alone and in combination with parthyroid extract significantly reduced plasma citrate levels. That the parathyroid extract had no effect

TABLE 2. PLASMA, BONE AND CO_2 ANALYSES OF MICE TREATED WITH ESTRONE AND PARATHYROID EXTRACT ALONE AND IN COMBINATION

Treatment	Body weight	Plasma citrate	Bone analyses		Specific activity* of expired CO_2
			Citrate concentration	% C^{14} in citrate	
	gm.	μg./ml.	mg./gm.		×10³
Control	28	74.1	7.17	.596	70.3
Estrone, 2 mg./kg.	27	44.1***	13.0**	.708***	103.
Parathyroid Extract 230 units/kg.	26	54.3	8.81	.918***	89.5
Estrone, 2 mg./kg. + Parathyroid Extract 230 units/kg.	27	50.5***	11.9**	.830	133.0

* Specific activity is defined as % C^{14} recovered per mg. CO_2

** $P < 0.01$

*** $P < 0.05$, that treated group is from same population as control as tested by the rank-sum method of Wilcoxon (6).

on citrate is not consistent with the results Martin *et al.* (2) have found in the dog. The present observation does agree, however, with an earlier observation from this laboratory (1) that parathyroid extract had no effect on plasma calcium in the mouse. Species or experimental differences may account for these conflicting results.

Estrone alone or in combination with parathyroid extract significantly elevated bone citrate levels. The parathyroid extract also tended to augment bone citrate concentration though the response was less than that for estrone and not statistically significant. Both the ovarian hormone and the parathyroid extract increased the incorporation of acetate C^{14} into bone citrate, although when both substances were administered together the effect was less than that observed in the group treated only with parathyroid extract. Though the amount of C^{14} found in bone citrate was elevated in the group treated with both materials the variability of the individual data was such that statistical significance of this difference from the control could not be demonstrated.

DISCUSSION

The assumption has been made that contamination of extracellular, isotopic citrate by labeled citrate from non-bony portions of the tibia and femur was minimal. This assumption is based on a preliminary experiment that demonstrated that 90 minutes after acetate-1-C^{14} administration the production of $C^{14}O_2$ had ceased. This would indicate that the tricarboxylic acid cycle in both bone and other tissues had exhausted the labeled exogenous substrate and that any labeled citrate recovered from the bone would have been trapped as the insoluble calcium complex, though some still might be present as soluble citrate in the extracellular compartment of bone. This experimental design, of course, does not exclude the inclusion in bone of isotopic citrate that arose from the synthetic activity of other tissues. However, if this did occur the amounts would be small because of the extensive dilution of the labeled material in the body fluids. For these reasons I feel the data provide a valid measure of bone citrate synthesis in the presence of the modifying hormonal materials.

Previous reports from this laboratory (1, 7) have demonstrated that estrone administration induces an increase in the accretion, exchange and resorption of Ca and P in bone. Such augmentation of bone ion turnover rates by estrone is also indicated in the data presented here for citrate. The increased incorporation of acetate-1-C^{14} into bone citrate in the estrone-treated group as compared to the control clearly indicates a heightened activity of the tricarboxylic acid cycle in this tissue. Concomitant with this enhanced bone citrate metabolism there was an apparent shift in the equilibrium between the soluble and insoluble citrate body pool in the direction of the latter as evidenced by the reduced plasma citrate level and the greatly augmented bone citrate concentration. One might conclude,

then, that two of the mechanisms whereby estrone increases bone citrate accretion may be the stimulation of the tricarboxylic acid cycle in the tissue and the enhancement of a "trapping" of citrate at the bone crystal surface.

Although bone Ca determinations were not carried out in this experiment, it was assumed that the Ca content of bone in the parathyroid extract treated group was not markedly different from that of either the control of the estrone treated animals. This assumption was based on the report of Munro *et al.* (8) that parathyroid extract (400 units/kg.) induced only a 4 per cent reduction in bone Ca in rats. Thus, a comparison of the parathyroid extract treated mice with the other groups seems valid.

Like estrone, parathyroid extract increased both the incorporation of labeled acetate into citrate and the oxidation of the substrate to $C^{14}O_2$. In the group treated with parathyroid extract as opposed to that treated with estrone there was no accumulation of citrate in bone nor a significant change in the plasma citrate level. One must presume that parathyroid extract did not enhance citrate "trapping" at the crystal surface and that the additional citrate formed was released to the extracellular fluid, passed to the circulation and was metabolized by other tissues or was excreted.

When both estrone and parathyroid extract were administered, the enhanced citrate metabolism peculiar to estrogen administration nearly masked the effects that the parathyroid extract might have shown. However, the estrone induced reduction in plasma citrate and increase in bone citrate concentration were not as great as were observed with the ovarian hormone alone. These differences, though not statistically significant, lend support to my previous demonstration of a parathyroid extract inhibition of the estrone induced increase in bone Ca accretion (1). The equivocal amount of C^{14} incorporation into bone citrate in this group cannot be easily interpreted, but the high specific activity of the $C^{14}O_2$ collected from this group of animals indicated, again, a primarily estrogenic response.

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EFFECT OF DOSAGE ON PLATEAUING IN ADULT FEMALE NORWAY RATS RECEIVING A GROWTH HORMONE CONTAINING PITUITARY EXTRACT[1]

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ABSTRACT

Nine groups of adult female Long-Evans rats, containing 8 to 10 rats each, were given daily injections of a growth hormone-containing extract of bovine anterior pituitaries at dose levels representing 1.28 to 12.8 mg. of growth hormone/kg./day. In each group there was initially a major resumption of growth, followed by establishment of a new growth plateau at a greater size. Group comparison of the weight gain during the initial and terminal 20 day periods gave t ratios ranging from 6.6 to 16.4 with p values ranging fron 2×10^{-7} to less than 10^{-10}. The time required for growth cessation ranged from 16 days at the lowest dose level to 200 days at the highest dose level. Weight gained prior to plateauing ranged from 21.6 ± 1.7 grams at the lowest dose level to 350.0 ± 14.4 grams (mean $\pm$ std. error) at the highest dose level. The length increments were relatively greater at low dose levels.

CHRONIC treatment of the plateaued adult rat with anterior pituitary growth hormone leads to an initial period of intensive growth followed by a period of reduced growth, followed in turn by growth cessation, although treatment is continued at the same dose level, either per rat or per unit weight (1, 2, 3, 4, 5, 6). This phenomenon of growth cessation or plateauing appears to occur in the same manner in rats given growth hormone which is pure by all currently available criteria, as in those receiving crude growth hormone-containing extracts (3, 5). Development of growth plateaus in rats on treatment with a growth hormone-containing extract of rat anterior pituitaries occurs in the same manner as in rats receiving an identical extract of bovine anterior pituitaries (6). Since the initial growth response is as great in senile rats as in young adults, plateauing cannot be due to a change of responsiveness with age (6). Apparently identical plateauing also occurs in rats receiving protein anabolic steroids (4, 7). Growth cessation while on a constant low dosage of growth hormone (4.0 mg./kg.) apparently is not a function of altered steroid production or steroid metabolism (8, 9). Since chronic treatment with growth hormone is not diabetogenic in the rat (5), diabetes mellitus cannot be the cause of plateauing.

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[1] Some of the data in this paper were presented in preliminary form at the 1955 Fall meeting of the American Physiological Society.

The present investigation was performed to determine the relationship between dose level of a growth hormone-containing anterior pituitary extract and both: (a) the time required for plateauing, and (b) the size attained prior to plateauing.

METHODS

The extract used in this investigation was prepared by the procedure we have previously described for a pH 10.5 extract (5) and contained all pituitary factors soluble at pH 10.0–10.5 and 0.9% NaCl. One ml. of the extract contained the extractives from 100 mg. wet weight of bovine anterior lobe tissue and was equivalent by the 10-day normal rat weight gain assay[2] to 3.2 mg. pure growth hormone. Throughout this paper we will designate dosage in terms of the mg. wet weight anterior lobe tissue represented by the daily dose. All of the extract was prepared as a single batch, stored frozen at −30° C in injection vials, and thawed just prior to use. Extracts of this type can be stored for several years under these conditions without measurable changes in growth promoting potency. Aliquots of the extract used in this investigation were assayed repeatedly before, during and after the experiments to be reported (10-day normal rat weight gain assay[2]) and no change in potency was detected.

Rats in all treated groups were injected once daily, subcutaneously, under aseptic conditions, at systematically varied sites along the dorsal surface. After each injection the rat was rewarded with 0.5 ml. imitation maple syrup. One control group was injected with pH 10.5 isotonic saline. The other control groups received no injections, but all controls received the same amount of syrup as the treated animals. The rats were 7-month old females from an all black strain developed from original Long-Evans stock. The experiments were performed in a semi-isolation room, maintained at 22 to 24° C, in which the rats had been raised from birth. All rats were maintained from weaning on Purina laboratory chow *ad lib.*

RESULTS

In the first experiments of this series, rats were treated at dose levels of 10 mg., 25 mg., and 100 mg./rat/day of the extract. Growth curves of these rats are shown in Figure 1. As can be seen, the 10 mg. and 25 mg. dosage led to sharp plateaus after approximately 20 days treatment. (Time for development of plateaus at 25 mg. dose level appears to be consistent from group to group as shown in Table 1.) As shown in Figure 1, the 100 mg. group had a sharp growth reduction after the first 20 days, but showed some growth throughout the 140 days of treatment. A reduction in growth rate after the first 20 days, with continued growth at a reduced rate for prolonged periods before a stable weight plateau is reached, appears to be a reproducible finding at dose levels of 50 mg. or higher/rat/day. (See Figs. 2 and 3; Tables 1 and 2).

We have previously shown that rats plateaued at one dose level will resume growth and again plateau at a greater size if dosage is increased (5). It therefore becomes of interest to inquire as to the size of rats on the final plateau at a given dose level, when that dose level is approached

[2] Mean λ (10 Assays) = 0.150 (λ = std. deviation/slope of log dose response curve).

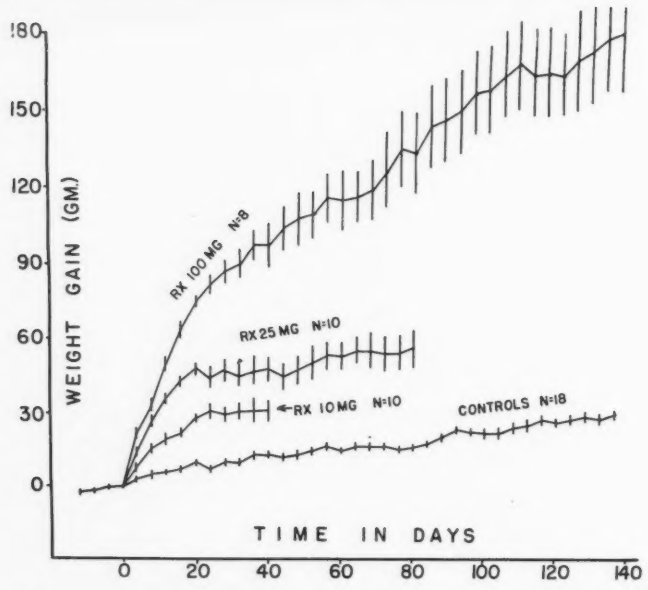

FIG. 1. Effect of different dose levels of pH 10.5 extract of bovine anterior lobes on plateauing treated groups 1, 3 and 5. Dosages expressed as mg. wet wt. of tissue represented by daily dose; 25 mg. is equivalent to 0.8 mg. growth hormone. Treatment was continuous from day 0 to end of graph in each case. Vertical bars extend 1 std. error of mean above and 1 std. error of mean below the mean at each point. For statistical analysis see Table 1.

TABLE 1. PLATEAUING* TIME AND WEIGHT AT DIFFERENT DOSES OF pH 10.5 ANTERIOR PITUITARY EXTRACT

Group	1	2	3	4	24	5	25	6	26
No. of rats	10	8	8	10	8	8	8	8	8
Daily dose†	10 mg./ rat	25 mg./ rat	25 mg./ rat	25 mg./ rat	25 mg./ rat	100 mg./ rat	100 mg./ rat	100 mg./ rat‡	100 mg./ 250 gm.
Days of plateau*	16	22	18	16	20	98‖	152	34‡	176
Wt. gain (gm.) over controls prior to plateauing	21.6 ± 1.7§	57.0 ± 4.8	38.1 ± 3.6	36.1 ± 2.5	48.8 ± 3.0	139.5‖ ± 18.0	205.7 ± 15.5	194.8 ± 24.4‡	350.0 ± 14.4
Wt. gain (gm.) over controls 1st 20 days	25.6 ± 2.4	54.6 ± 4.7	40.0 ± 3.2	38.1 ± 2.8	48.8 ± 3.0	68.7 ± 2.3	76.0 ± 2.7	54.6 ± 4.7	75.7 ± 2.8
Wt. gain (gm.) over controls last 20 days	− 6.7 ± 4.3	− 2.9 ± 2.7	− 5.7 ± 3.9	1.1 ± 3.2	1.3 ± 2.5	9.2 ± 7.5	5.6 ± 3.3	− 3.5 ± 5.4	− 2.4 ± 4.2
t 1st 20 days vs. last 20 days	6.6	10.6	9.0	8.0	12.2	7.5	16.4	8.1	15.3
p	2×10^{-7}	10^{-10}	10^{-10}	10^{-9}	10^{-10}	10^{-9}	10^{-10}	10^{-9}	10^{-10}

* Plateaus to criterion of less than 4 gm. gain over controls in 16 days.
† Wet wt. of pituitary tissue represented in daily dose.
‡ Brought to this dose in 3 steps over 225 days.
§ S.E. of Mean.
‖ This was not the final plateau and was probably spurious.

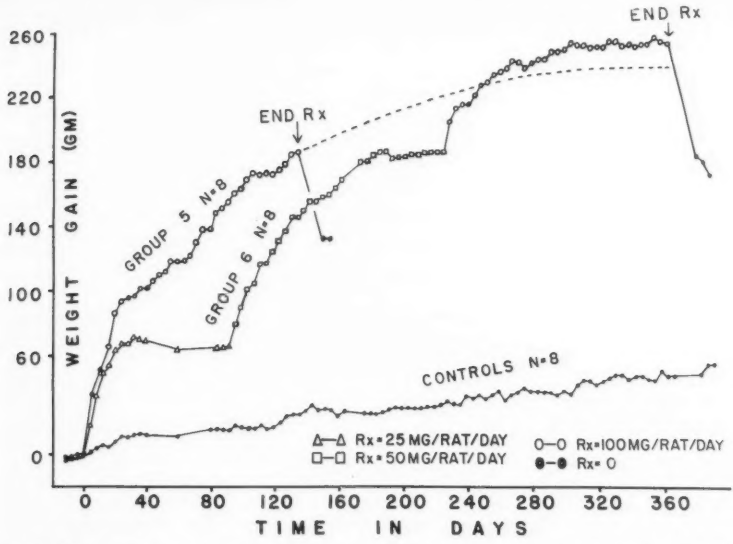

FIG. 2. Effect of stepwise increments in pH 10.5 extract up to a dosage of 100 mg. compared with employment of this dosage from beginning of experiment. For statistical analysis see Table 1. Daily injections given from day 0 to points indicated.

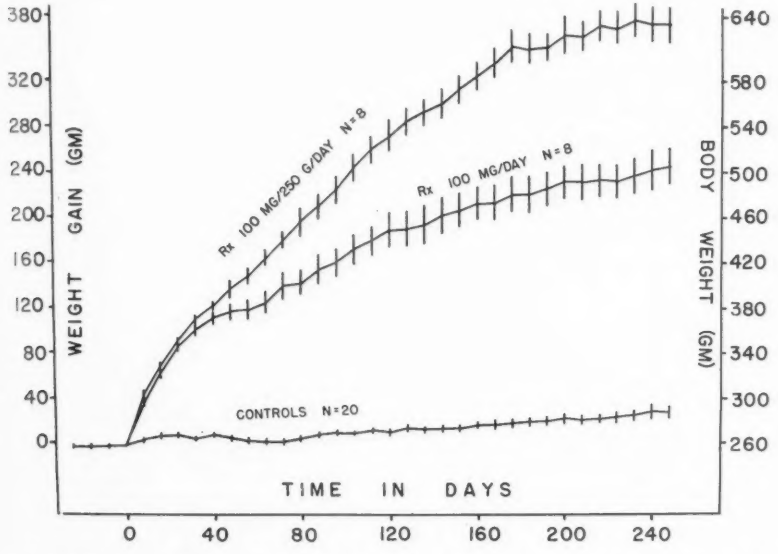

FIG. 3. Effect of high dosage, Groups 23, 25 and 26. Expression of dosage and significance of vertical bars are the same as in Figure 1. Daily injections were given from day 0 through day 247.

TABLE 2. MEAN GAIN (GM.) OF TWO TREATED GROUPS (100 MG./250 GM. BODY WT.)* MINUS CONTROLS DURING SUCCESSIVE 20 DAY PERIODS

Group	Number		20 day pretreat.	Gain during 20 day periods of treatment								
	Treat.	Cont.		1st	2nd	3rd	4th	5th	6th	7th	11th	12th
26 minus 23	8	20	1.1±2.0†	75.7 ±2.8	38.0 ±3.3	35.6 ±8.4	40.1 ±6.8	38.5 ±4.2	33.2 ±4.2	22.3 ±4.5	6.4 ±4.8	−2.4 ±4.2
29 minus 28	8	14	1.7±1.7	79.3 ±3.9	33.1 ±6.0	37.9 ±4.2	36.3 ±3.7	33.7 ±3.8	30.9 ±3.7	20.7 ±2.9	—	—

* Dosage given in mg. wet wt. anterior lobe tissue represented by the daily dose. This dose is equivalent to 3.2 mg. growth hormone per 250 gm. body weight per day.
† Mean weight gains of treated minus controls for 20 day periods are reported plus or minus the standard error of the difference.

stepwise, compared with the final plateaued size of rats at the same dose level when that dosage is administered from the beginning of treatment. Figure 2 shows the growth curves of a group of rats started at the same time as those in Figure 1, in which the dosage was increased stepwise from 25 mg./rat/day to 100 mg./rat/day. The Group 5 animals of Figure 2 are the same 100 mg. rats as in Figure 1. The dashed line continuing from the Group 5 curve is our estimate of these rats' growth had treatment been continued. According to this estimate, the final plateaued level of Group 5 and Group 6 rats would not have differed meaningfully. That this estimate was reasonably accurate is shown by the growth curve of the 100 mg. group in Figure 3. Although the experiments in Figure 3 were performed after those in Figure 2 were concluded, we consider them to be reasonably comparable since the 100 mg. group of Figure 3 (Group 25) had a weight gain at the end of 140 days of 191 ±16.8 gm. compared with 183.6 ±22.6 gm. for Group 5 rats of Figure 2. The final weight gain of the Group 25 rats was 244.1 ±16.8 gm. compared with the estimated 240 gm. of the Group 5 rats and 250.1 ±36.1 gm. for the rats in Group 6.

The final length of the rats in Group 6 was also essentially identical with that of the Group 25 animals (Table 3). It is of interest that the Group 5 rats lost 30.5% of the weight they had gained during the first 16 days after treatment was terminated, compared with a loss of 28.0% in

TABLE 3. LENGTH OF FEMALE RATS TREATED WITH pH 10.5 ANTERIOR PITUITARY EXTRACT

Group No.	No. rats	Daily dose	Length (cm.)		
			Nose-anus	Anus-tail tip	Total
23	20	None	22.02 ±0.15*	19.09 ±0.22*	41.11 ±0.33*
26	8	100 mg./250 gm.	26.63 ±0.22	21.19 ±0.17	47.81 ±0.26
25	8	100 mg./rat	25.53 ±0.43	20.72 ±0.33	46.25 ±0.52
6	8	100 mg./rat†	25.28 ±0.27	20.74 ±0.21	46.02 ±0.39
24	7	25 mg./rat	24.27 ±0.29	20.30 ±0.20	44.57 ±0.38
35‡	15	None	13.57 ±0.12	9.39 ±0.30	22.95 ±0.38

* Mean ±Standard Error of Mean.
† Reached a dose of 100 mg./rat/day in 3 stages.
‡ This group was composed of 30 day old, 61 gram infant females; all other groups were adult.

the Group 6 rats. This striking weight loss of plateaued or nearly plateaued rats when taken off treatment has been previously reported by Lee and Schafer (10) and by Evans and Simpson (11), and constitutes clear-cut evidence that growth hormone is still producing an effect although the animals are not still growing. Even more striking than the weight loss is the loss of strength and vigor which occurs after cessation of treatment in rats which have reached a marked giantism. The pituitary giants are always extremely muscular and appear distinctly more vigorous than their controls. Within only two weeks after cessation of treatment these animals become extremely weak and emaciated, appear to require great effort to stand and, in general, show a picture strikingly similar to advanced Simmonds' disease in man.

Since at dose levels of 25 mg./rat/day (0.8 mg. growth hormone) or less there is sharp plateauing within approximately 20 days, whereas at dose levels of 50 mg./rat/day or higher there is growth for much longer periods, and the plateau appears to be reached more asymptotically, it is conceivable that the animals at the higher dose levels are merely growing to such size that their plasma and tissue fluid concentrations of growth hormone become the same as in the 25 mg./rat/day group. To clarify this point the experiments reported in Figure 3 were performed. In these experiments, one group received 100 mg./rat/day and another group received 100 mg./250 gm. body weight/day. The initial dosage was essentially the same in both groups since the initial mean weight was 260 gm. The 100 mg./rat/day group gained 244.1 ± 16.8 gm. in 240 days of treatment compared to 373.3 ± 16.2 gm. in the 100 mg./250 gm./day group. Since 25 mg./rat/day initially gives a near maximal growth, the 100 mg./250 gm./day group reached a flat plateau at a dose level per unit body weight four times that required for near maximal response in previously untreated animals. Furthermore, at this high dosage there was still a marked change in the shape of the growth curve after the first 20 days (Fig. 3). Although the animals in this experiment appeared to be in excellent health throughout the experiment, there is always the outside possibility of some illness, giving no readily observable signs, which could alter a growth curve. Consequently, since we consider the data on the 100 mg./250 gm./day group to be of critical importance, another group was run at this dose level for 140 days. In Table 2 the growth of treated minus controls during 20 day periods is given for each of the 100 mg./250 gm./day groups. As can be seen, the growth of these two groups, including the reduced rate of growth after the first 20 days, was essentially identical.

Table 1 gives the time required to plateau (the criterion is not more than 4.0 gm. mean gain of treated rats over controls in a 16-day period), and the mean weight gain prior to plateauing for all groups in the present investigation.

Data on the length of rats at different dose levels are given in Table 3.

Since growth is a three-dimensional process, one would assume that if growth were truly symmetrical, adult wt./infant wt. divided by adult ln.^{3}/infant ln.^{3} would give a ratio of 1. When normal adults (Group 23) were compared to infants (Group 35) in this manner, however, the ratio was 1.11 ± 0.054[3] (P vs $1 = 0.02$), indicating some degree of visceromegaly in normal growth. (Nose-anus lengths were used for this comparison.) The same comparison of Groups 26 and 23 gives a ratio of 1.24 ± 0.065[3] (P vs $1 = 0.0007$), suggesting a further development of visceromegaly in the pituitary giant rat. This increased visceromegaly is not, however, as great as this ratio would indicate since Lee (10) has shown that the percentage carcass fat is reduced by growth hormone treatment, thereby increasing the specific gravity of the tissue. Comparison of Group 24, treated at a low dosage, to Group 23 gives a ratio of 0.90 ± 0.033[3] (P vs $1 = 0.003$), suggesting that at a low dose level, the length increment is relatively greater than the weight increment. (It would be of interest to know whether the animals in Group 26 had a length as great as the plateaued length of Group 24 by the time they had a weight gain equal to the plateaued gain of Group 24. We plan to make such measurements in the near future.)

DISCUSSION

We interpret the above data as showing, at a high probability level: 1) That plateauing occurs under the conditions of this investigation at all dose levels up to and including 100 mg./250 gm. body wt./day (equivalent to 3.2 mg. growth hormone/250 gm. body wt./day); 2) That the weight gained prior to plateauing is nearly identical, at a given dose level, irrespective of whether that dosage is reached by stepwise increments or is the initial dosage; 3) That at dose levels of 25 mg./rat/day or below, plateauing occurs in approximately 20 days; whereas, at dose levels of 50 mg./rat/day or greater, a much longer period is required for plateauing.

We further interpret these data as suggesting that there may be multiple factors involved in plateauing of rats on treatment with a growth hormone-containing extract of anterior pituitaries and that one factor or factors may be relatively more important at dose levels of 25 mg./rat/day or less and another factor or factors may be relatively more important at higher dose levels. It is possible that 25 mg./250 gm./day (0.8 mg. growth hormone/250 gm./day) may represent the approximate dosage at which a single daily injection maintains an average plasma and tissue fluid growth hormone concentration equivalent to that maintained by the adult rat's own pituitary and that 16–24 days represents the time required for the

[3] Std. Error of mean. These Std. Errors established by successive application of:

1. $$\sigma \frac{\bar{x}_1}{\bar{x}_2} = \frac{\bar{x}_1}{\bar{x}_2} \cdot \sqrt{\left(\frac{\sigma \bar{x}_1}{\bar{x}_1}\right)^2 + \left(\frac{\sigma \bar{x}_2}{\bar{x}_2}\right)^2}$$

2. $$\sigma \bar{x}^N = \sqrt{N} \cdot \sigma \bar{x} \cdot \bar{x}^{N-1}$$

control mechanism regulating growth hormone production to act in shutting off endogenous production.

In the higher dose ranges, the fact that the 100 mg./rat/day groups gained about ⅔ as much as the 100 mg./250 gm./day groups, which were receiving 2.5 times as high a dose level on their final plateau, suggests that we are dealing with some type of parametric forcing. The increase in cytoplasmic volume per unit membrane area in muscle fibers, which would be anticipated on the basis of Morpurgo's (12) observation that the fiber count of muscles fails to increase in normal growth, constitutes an interesting possibility for a parametric limit to growth. Preliminary work in this laboratory indicates, however, that the mean diameter of skeletal muscle fibers in the pituitary giant rat is essentially identical to that of the normal adult, even though the cross-sectional area of the entire muscle may be twice as great. We will present detailed data on muscle fiber diameter in a subsequent paper.

Selye (1) has found that in his laboratory, rats plateaued on growth hormone treatment showed increased incidence of respiratory, cardiovascular and renal disease. We have likewise observed that rats, plateaued at a high dose level for many months, show an increased incidence of a non-bacterial respiratory disease, with low or zero infectivity, and showing many of the signs and sequelae of bronchial asthma. We have not, however, observed increased incidence of such disease until the rats have been plateaued on treatment for 6 to 8 weeks. Approximately 80% of the rats in Groups 26 and 29 were free of rales and had pink lungs which collapsed upon opening the thorax at autopsy. We are in the process of measuring oxygen content of arterial blood in a group of rats plateaued at a high dose level compared with split litter controls and have, thus far, found only slight differences. Detailed data will be presented when this investigation is completed. In addition, we placed 3 rats showing the above respiratory condition prior to treatment, on 100 mg./250 gm./day. During the first 20 days of treatment, these animals had a mean gain of 85.7 ± 2.1 gm. over their controls—a response slightly greater than that of the animals in Groups 26 and 29. Preliminary data have been presented from our laboratory (13) showing that the cardiac output of the plateaued pituitary giant rat (closed chest, direct Fick by cardiac catheterization) is as great per 100 gm. body weight as in split litter controls.

Detailed search of the abdominal and thoracic cavities of the 8 animals in Group 26 disclosed one grossly observable periarteritis nodosa lesion, compared with 2 lesions in the 20 split litter controls in Group 23.

No measurements of renal function in plateaued growth hormone treated rats have been made in our laboratory. Gershberg *et al.* (14), however, have found marked increase in C_{In}/M^2 and in T_M/M^2 (14) for several substances in a case of human acromegaly of many years standing. These observations suggest that the well known acute renotrophic action of

growth hormone persists in the presence of a prolonged high growth hormone titer, at least in man.

We have previously shown (15) that daily administration of large amounts of plasma from rats plateaued at high dosage fails to reduce the initial response of rats to small dosage of the extract. Thus it appears that the plasma of rats plateaued at high dosage does not contain an appreciable titre of a growth hormone inactivating antibody.

It is our tentative hypothesis that both plateauing and the various non-malignant hypertrophic diseases which appear in rats that have been on plateaus at high dosage for prolonged periods may reflect a basic alteration in protein metabolism.

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THE EFFECT OF HYPOTHALAMIC LESIONS ON OVARIAN FUNCTION IN THE EWE[1]

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ABSTRACT

In 20 regularly cycling ewes, stereotaxic lesions were placed in various parts of the hypothalamus, and changes in estrous behavior, ovarian cycles, pituitary gonadotrophin content as well as adrenal and thyroid function were studied. Destruction of the pituitary stalk and adjacent ventral hypothalamus was associated with absence of both ovarian and behavioral cycles (4 animals). Destruction of an area immediately above the anterior median eminence inhibited estrous behavior without affecting ovarian cycles (3 animals). Extensive lesions in other parts of the hypothalamus (13 animals) had no effect on these parameters. In two animals with stalk lesions peripheral 17-hydroxycorticoid levels were not elevated following stress but in none of the animals was there significant adrenal or anterior pituitary atrophy. The FSH content of the anterior pituitary was somewhat depressed without a definite change in the LH content in animals with stalk lesions. Animals with lesions which did not involve the stalk had normal pituitary gonadotrophin content. Normal sheep which have entered the anestrous phase of their yearly cycle had a lower pituitary LH content than ewes during the polyestrous part of the year.

IN SEASONALLY polyestrous mammals like the sheep, the well established effect of light on pituitary secretory activity is presumably mediated through neural mechanisms integrated in the hypothalamus. In the human female, amenorrhea is a common symptom of ventral hypothalamic disease (1), and in rats (2), cats (3, 4), guinea pigs (5) and rabbits (3) ovarian atrophy is associated with experimental destruction of some hypothalamic areas. The effect of anterior hypothalamic lesions on sexual behavior in ewes has been reported (6), but inhibition of pituitary gonadotrophin release by ventral hypothalamic lesions has not been clearly documented in this species.

MATERIALS AND METHODS

The present report deals with data obtained during two experiments in two consecutive years. In both studies, sexually mature white-faced ewes were used. Throughout the

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experimental period, they were maintained on pasture and, when necessary, fed good quality alfalfa hay *ad libitum*. In each series the animals were checked daily for receptivity to the male by placing them with vasectomized or aproned rams from the first of August until the animals were sacrificed. Electrolytic lesions were placed stereotaxically in various portions of the hypothalamus by a previously described technique (6). All but one animal had two or more heat periods pre-operatively; one animal had only one pre-operative heat.

In the first experiment (series 1), laparotomies were performed on all animals and the appearance of the ovaries noted two months post-operatively. Immediately after laparotomy, peripheral venous blood specimens were obtained for measurement of 17-hydroxycorticoid levels. The heparinized samples were centrifuged immediately and the plasma stored in the frozen state. Seventeen-hydroxycorticoid determinations were performed by the method of Silber and Porter (7). Thereafter, daily checks for heat continued for two more months, at which time the animals were sacrificed. Twenty-four hours before autopsy, the animals were given approximately four μc. of I^{131} intraperitoneally. At autopsy, the thyroid, adrenals and ovaries were removed, dissected and carefully weighed. A separately weighed portion of the thyroid gland was placed in a vial containing concentrated sodium hydroxide and subsequently counted in a scintillation counter. The remainder of the thyroid, the ovaries and the adrenals were fixed in Bouin's solution, sectioned, stained with hematoxylin and eosin and examined. In five animals, the whole pituitary was removed, weighed and similarly prepared for histological examination. In the remaining eight animals, the whole pituitaries were immediately frozen in dry ice, the posterior lobes removed and the anterior lobes lyophilized. The dried anterior pituitary powder from each animal was assayed for follicle stimulating hormone (FSH) and luteinizing hormone (LH) according to procedures previously described (6).

The hypothalamus was carefully removed, fixed in 10% formalin solution and serially sectioned at 10 micra. Every 25th section was mounted, stained with hematoxylin and eosin, and used to reconstruct the hypothalamic lesions. The lesion in each animal was sketched on a diagram of a sagittal section of the sheep hypothalamus.

The lesion reconstruction permitted dividing the animals into groups on the basis of common areas of damage. In this experiment, four ewes had an area of destruction which included a portion of the pituitary stalk and adjacent median eminence. One animal had a lesion just above the median eminence, and the remaining four had lesions of the massa intermedia and dorsal hypothalamus. Five unoperated normal sheep served as controls.

In the second experiment (series 2), the animals were checked daily for heat until they were sacrificed approximately two and one-half months post-operatively. Data on thyroid uptake of radioactive iodine and 17-hydroxycorticoid blood levels were not obtained in this series of animals.

The adrenals, thyroid and ovaries were weighed, fixed, sectioned and histologically examined in the same manner as the previous series. FSH and LH content of the pituitaries were determined as before. Following reconstruction of the hypothalamic lesions, the animals were divided on the basis of the areas destroyed. Two of these animals had lesions in the anterior hypothalamus above the median eminence. Nine sheep had lesions in various other parts of the hypothalamus, sparing the stalk region, the median eminence and the region immediately above it. Twelve unoperated animals served as normal controls.

RESULTS

The data obtained in the individual animals are summarized in Table 1 (series 1) and Table 2 (series 2). The sheep in series 1 were subjected to laparotomy while the control animals were still cycling regularly, but this

TABLE 1. EFFECT OF HYPOTHALAMIC LESIONS ON REPRODUCTIVE ACTIVITY, CONTENT OF PITUITARY GONADOTROPHINS AND OTHER ENDOCRINE FUNCTION IN SERIES 1 SHEEP

Sheep No.	No. heats		Ovarian histology during cycles	LH mg. equiv. per ant. lobe	FSH A.U. per ant. lobe	Wt. of whole pituitary, mg.
	pre-operative	post-operative				
Stalk lesion						
803	3	0	A	—	—	—
812	2	0	A	5.2	0.74	630
824	3	0	A	8.3	0.25	620
825	2	0	A	—	—	620*
Mean ±S.E.				6.8	0.50	623 ±3.0
Anterior lesion—heat absent						
801	1	0	C	10.1	0.76	710
Control lesion						
805	2	1	C	—	—	780
808	4	2	C	9.9	1.75	740
816	4	3	C	16.8	1.49	650
822	2	5	C	—	—	910
Mean ±S.E.				13.4	1.62	770 ±54
Intact normals						
814		2†	C	6.1	1.02	890
806		2	C	—	—	810
820		2	C	—	—	1000
807		3	C	5.9	1.05	650
819		2	C	6.1	1.25	1000
Mean ±S.E.				6.0 ±0.12	1.11 ±0.07	870 ±66

* Histologically small infarct.
† No. of heats occurring in intact normals after date of last operation.
A.U. = Armour units.
S.E. = standard error.
A = acyclic ovary no corpora lutea; only small follicles.
C = cyclic ovary corpora lutea present with large follicles.

group of animals was not sacrificed until all control animals had become anestrous. In series 2, on the other hand, the animals were killed while the controls were still cycling regularly. Therefore, as far as the observations on ovarian histology and cycles are concerned, the laparotomy data in series 1 are comparable to the autopsy data in series 2. Combining the two series, the ovaries were acyclic; that is, they showed only small to medium sized follicles and no corpora lutea in four ewes. All four of these animals had lesions involving the pituitary stalk, although the stalk was not completely destroyed in any of these animals (Figure 1). In all cases, there was some destruction of the adjacent anterior median eminence. In the remaining 16 ewes the ovaries contained corpora lutea and/or follicles more than 7 mm. in diameter. In three of these 16, there were no heat periods post-operatively. In each of these three animals, there was destruction of a small area above the middle portion of the median eminence in front of the pituitary stalk. In 13 animals, one or more normal cycles occurred post-operatively and the ovaries showed cyclic changes. The

TABLE 2. EFFECT OF HYPOTHALAMIC LESIONS ON ESTROUS CYCLES AND PITUITARY GONADOTROPHIC CONTENT OF SERIES 2 SHEEP

Sheep No.	No. heats		Ovarian histology during cycles	Lg mg. equiv. per ant. lobe	FSH A.U. per ant. lobe	Wt. of whole pituitary mg.
	pre-operative	post-operative				
Anterior lesion—heat absent						
905	2	0	C	13.3	1.54	750
911	3	0	C	31.5	1.86	1460
Mean ±S.E.				22.4	1.70	1105
Control lesion						
901	2	3	C	10.4	0.90	590
906	2	1	C	15.5	1.18	890
907	2	3	C	—	—	—
912	2	4	C	22.7	1.39	1050
916	2	4	C	19.9	4.06	1070
932	2	2	C	13.4	1.94	720
938	4	3	C	18.6	1.12	980
946	3	5	C	17.9	1.36	1200
948	2	4	C	23.2	<0.5	1370
Mean ±S.E.				17.7 ±1.56	1.56 ±0.38	983.7 ±89
Intact normals						
902		3*	C	22.9	1.96	1490
903		4	C	21.0	3.08	1220
904		4	C	22.7	1.94	1110
908		5	C	14.0	1.08	1010
915		5	C	22.1	2.89	1310
923		4	C	26.2	1.92	1520
924		5	C	15.7	1.14	1150
930		4	C	23.0	2.10	1040
931		5	C	14.3	0.73	900
936		5	C	18.6	2.85	930
949		3	C	28.8	1.64	1000
950		4	C	19.8	<0.5	1020
Mean ±S.E.				20.8 ±1.31	1.82 ±0.24	1141.7 ±59

* No. of heats occurring in intact normals after date of last operation.
S.E. = standard error.
A.U. = Armour units.
C = cyclical ovaries as indicated by the presence of corpora lutea and large follicles.

lesions in these animals involved variously the pre-optic region, the area above the median eminence, the massa intermedia and the dorsal hypothalamus immediately adjacent to it (Figure 2). These lesions spared the small area above the median eminence which was damaged in the animals without heats as well as the pituitary stalk and median eminence.

The peripheral levels of 17-hydroxycorticoids for the animals in series 1 were not significantly changed following the stress of laparotomy except in two animals with lesions. In both these animals measurable values could not be demonstrated. They had lesions which involved the infundibulum but extended slightly more anteriorally in the median eminence than the lesions in the other two animals with pituitary stalk damage. The values for the plasma 17-hydroxycorticoids of these latter two were 6.3 and 10.0 mcg. per 100 ml. These levels can be compared with a value of 5.3 ± 1.6

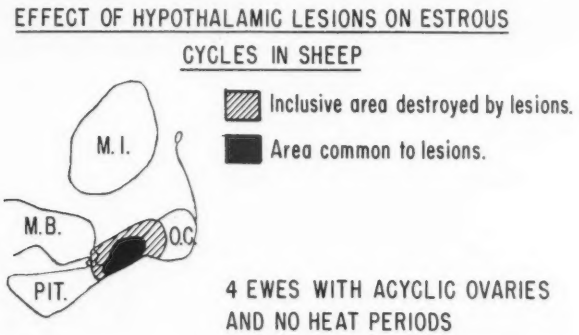
EFFECT OF HYPOTHALAMIC LESIONS ON ESTROUS CYCLES IN SHEEP
Inclusive area destroyed by lesions.
Area common to lesions.
M. I.
M.B.
O.C.
PIT.
4 EWES WITH ACYCLIC OVARIES AND NO HEAT PERIODS

FIG. 1. Diagram projected on the mid-sagittal section of the hypothalamus illustrating the areas in which lesions were placed. M.I., massa intermedia; M.B., mammillary body; O.C., optic chiasm; Pit., pituitary.

mcg. per 100 ml. (mean $\pm$ standard error of the mean) for the intact normals and 11.7 ± 2.4 for those sheep with control lesions. Thyroid and adrenal weight were not significantly different among groups nor were there changes in histological appearance. The mean adrenal weight in the four animals with stalk lesions was 2.48 ± 0.14 gm.; in the normal control animals it was 2.94 ± 0.19 gm,; and in the animals with lesions sparing the pituitary stalk it was 3.13 ± 0.18 gm. Mean thyroid weight in the animals with stalk lesions was 2.67 ± 0.67 gm., while in the normal controls it was 2.21 ± 0.30. The range in the other animals in this series was from 2.10 to 3.02 gm. Thyroid uptake of radioactive iodine for the sheep with stalk and median eminence lesions ranged from 12.5 to 17.5 percent of injected dose; those with control lesions ranged from 15.2 to 25.7 and the mean

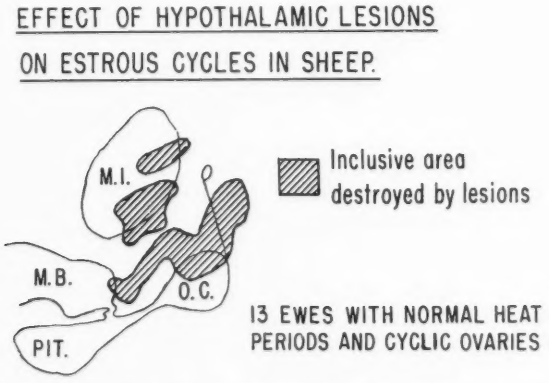

FIG. 2. Diagram illustrating the areas in which lesions were placed in the control animals. Note that in all cases the mid-ventral median eminence and stalk are intact. M.I., massa intermedia; M.I., mammillary body; O.C., optic chiasm; Pit., pituitary.

value for the intact animals was 20.3 ±4.2. No significant differences exist among these values.

In series 2, the mean thyroid weight in the two animals with lesions in the anterior hypothalamus was 7.43 gm. and the mean adrenal weight was 3.90 gm. In the nine animals with lesions excluding this area and the pituitary stalk, mean thyroid weight was 8.01 ± 1.91 gm. and adrenal weight was 3.77 ±0.29 gm. These values are comparable to 6.56 ±0.62 gm. for the thyroid and 4.45 ±0.28 gm. for the adrenals in the 12 normal controls. No abnormality of thyroid or adrenal histology was observed in the animals with lesions in this series.

Because the animals in series 1 were killed after the controls had become anestrous, it is essential to treat the data on pituitary LH and FSH content separately in each of the two series. In series 1 the pituitary weight of the animals with stalk lesions was definitely less than that in the control animals with lesions as well as the intact normals. The mean weight of the whole pituitary in the animals with stalk lesions differs from the mean pituitary weight of the normal controls at the 5% level. The pituitary was examined histologically in one of these animals. A very small area of anterior pituitary infarction was present near the capsular wall, but most of the anterior pituitary was intact. On the other hand, extensive posterior lobe atrophy was present.

The individual values for LH and FSH content of the individual pituitaries in series 1 are shown in Table 1. The content of LH in the pituitaries of the two animals with stalk lesions is of the same order of magnitude as that found in the other animals with lesions and that found in the normal controls, while the FSH values are low.

In series 2, pituitary weights were not significantly reduced in any of the animals with lesions. The pituitary FSH values and particularly the pituitary LH values were higher in the control animals of this series than in the controls of the previous series. The former animals were all killed while still cycling normally. None of the lesions in this series appeared to have any significant effect on the FSH and LH content of the anterior pituitary.

DISCUSSION

In the present experiments, the four ewes with lesions in the pituitary stalk and adjacent median eminence all showed absent heat periods and no histological evidence of cyclical ovarian activity. It seems clear, therefore, that release of pituitary gonadotrophins was inhibited in these animals. The question of whether this was due to interruption of some specific neurohumoral regulating mechanism or to interference with the pituitary blood supply can be answered only indirectly. Since most of the pituitaries were saved for gonadotrophin bioassay, only one of these pituitaries was examined histologically. There was a small infarct involving no more than 3% of the total anterior lobe in the gland from this animal. On the other

hand, there was extensive atrophy of the posterior pituitary. This suggests that the relatively low pituitary weight in the animals with inhibited ovarian cycles was mainly due to a degeneration of the posterior lobe which regularly follows interruption of the supra-optical-hypophyseal nerve fibers (8). It seems unlikely that the observed depression of ovarian activity was the result of a pituitary hypofunction associated with nonspecific vascular insufficiency for several reasons. The pituitary stalk was not completely interrupted in any of these animals. Even complete stalk section in the sheep does not lead to total infarction of the anterior lobe because a portion of this lobe is supplied by anastomotic branches from the artery to the posterior lobe (9). Finally, in other species such as the dog and rat, more than 70% of the anterior pituitary must be destroyed before significant depression of gonadal activity occurs (10, 11).

Three ewes, one in series 1 and two in series 2, showed absence of behavioral heat but normally cyclic ovaries. The lesions in these animals involve the area previously reported to be concerned with sexual behavior in this species (6).

Important control data is supplied by the 13 sheep in the present series with hypothalamic lesions sparing the "sex area" and pituitary stalk. In all of these animals, normal cycles continued after lesion production and the ovaries were normally cyclic. That such extensive lesions failed to affect either the behavioral or the endocrine manifestations of the estrous cycle is strong additional evidence for the specificity of the hypothalamic centers involved in regulating these two phenomena.

It is interesting to compare the values for pituitary gonadotrophin content in the normal animals of series 1 killed after they became anestrous, with those in series 2, obtained while the animals were still cycling regularly. Although the FSH values in the former animals are somewhat lower than those in the latter, there is considerable overlap in the two series. On the other hand, pituitary LH activity was clearly lower in the intact anestrous ewes than in the intact ones sacrified during estrus. Furthermore, in one series 1 ewe, No. 816, LH potency was the highest of all others in this group. This animal had a lesion in the massa intermedia and contained one healthy corpus luteum, whereas the ovaries of the other sheep in this series which contained pituitaries of low potency were all acyclic at the time of sacrifice. In only one other study (12) have changes in the concentration of pituitary gonadotrophins been related to the breeding season of sheep. Pituitary gonadotrophic potency was reported to be greater in anestrous ewes than in those examined during estrus. Since this investigation was based on weight changes produced in the testes of two-day-old male Leghorn chicks to a measured amount of anterior pituitary tissue, it did not differentiate between LH and FSH content.

In the animals with stalk lesions in series 1, FSH content was lower than that in the controls while LH content was in the same range. This sug-

gests that hypothalamic lesions of this type depress FSH content without an appreciable effect on LH content. Since these data are available for only two animals, any conclusions must be regarded at present as highly tentative. On the other hand, it is interesting that in both series, lesions which inhibited behavioral heat without affecting ovarian cycles did not alter gonadotrophin content when the values are compared to their respective controls; furthermore, in all 13 animals with lesions in various portions of the hypothalamus, no detectable change in FSH and LH content occurred.

The 17-hydroxy-corticoid values in two animals with pituitary stalk lesions suggest inhibition of the pituitary response to stress. The absence of this response in animals with adrenal glands of normal weight and histological appearance has been reported in a number of animal species as an effect of median eminence lesions (13). On the basis of weight, histology and radioiodine uptake of the thyroid, none of the lesions in the two series had a significant influence on thyroid function.

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SOME PROPERTIES OF AN OXYTOCIC SUBSTANCE IN EXTRACTS OF SEMEN

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ABSTRACT

Oxytocic (uterine-stimulating) substances have been demonstrated in extracts of both human and ram semen. A modification of the method for extracting oxytocin from blood (Bisset, Walker 1954) was used (14). Some physicochemical and pharmacological properties of these extracts were investigated, and the evidence suggests that the active uterine-stimulating material in human semen differs from that in ram semen, and neither is considered to be oxytocin, potassium, acetylcholine, 5-hydroxytryptamine, histamine, hypertensin (angiotonin or angiotensin), substance P, kinin (bradykinin, kallidin, pain producing substance, substance U, substance Z), vasopressin (antidiuretic hormone), pepsitensin, pepsitocin, pepsanurin or the oxytocic substance found in blood.

AN OXYTOCIC[1] (uterine-stimulating) substance (O.S.) has been extracted from blood taken from human subjects (1, 2 and 3), cows (4), goats (4), sheep (5) and rats (6). This substance is not considered to be oxytocin or any of the many substances which are known to stimulate the rat uterus, viz. potassium, acetylcholine, 5-hydroxytryptamine (HT), vasopressin (antidiuretic hormone), substance P, kinin (bradykinin, kallidin, substance U, substance Z), pain producing substance (P.P.S.), pepsitensin, pepsitocin, pepsanurin or hypertensin (angiotonin or angiotensin).

It has been reported that seminal fluid from the human, sheep and goat stimulates uterine contractions both *in vitro* and *in vivo* (7, 8, 9 and 10). Eliasson (10) has commented that in man, and probably also in sheep, coitus and deposition of semen in the vagina seem to be essential in order to evoke passive migration of spermatozoa. Others have attributed the increase in uterine motility during sexual stimulation to the liberation of oxytocin (11 and 12).

We previously observed that copulation did not increase the level of oxytocin in extracts of ewes' blood (5): this, we realise, might be attributable to inadequacy of our method of extraction and/or assay.

An alternative explanation for the increased uterine motility observed during coitus might, in part at least, be due to the semen possessing oxytocic activity. Accordingly, we decided to apply the method we have been using for extracting O.S. from blood to seminal fluid to see whether an oxytocic substance could be extracted from human and ram semen.

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[1] "Oxytocic" is herein used as an operational term pending specific characterization.

We report in this paper that extracts of human and sheep seminal fluid possess oxytocic activity, and we describe some experiments which clearly indicate that the active substance(s) in these extracts differs from a number of known uterine stimulants.

METHODS

Preparation of the extracts

Samples of semen were collected from healthy men, and from rams by electrical stimulation of ejaculation (13), and were added immediately to acid-alcohol and extracted according to the author's modification (1) of Bisset and Walker's method (14) for extraction of oxytocin from blood.

The possibility of K^+, HT or acetylcholine in the extracts acting as interfering uterine stimulating substances was eliminated as described in an earlier paper (15), except that Dibenzyline was used instead of Dibenamine. All extracts were treated with sodium thioglycollate (0.01 M at pH 7.5 for 30 minutes) in order to inactivate oxytocin (16 and 17). Mepyramine was added to the organ bath to eliminate possible effects due to histamine (15).

Methods of assay

(a) *Rat uterus.* All extracts were assayed against Pitocin (Parke, Davis and Co.) on the isolated sensitized uterus of virgin Wistar strain rats as described by Holton (18) and Gaddum, Peart and Vogt (19).

(b) *Isolated rabbit and guinea-pig intestine.* These preparations were used to assay *ram* semen extracts: portions of the upper ileum were suspended in Mg^{++}-free Tyrode's solution and longitudinal muscle contractions were recorded (15).

Stability to heat at various pH

Aliquots of *ram* semen extract were heated in a water bath at 100° C with condenser as follows: 7.5 min. at pH 7.5; 60 min. at pH 7.5; 7.5 min. at pH 0.40; 7.5 min. at pH 11.5. Control samples of the extracts were kept at room temperature at pH similar to the experimental aliquots. Immediately after boiling the samples were chilled to 0° C and the pH was, where necessary, readjusted to 7.5. In addition, part of the extracts was kept at pH 2.5 at room temperature for 60 minutes and tested at both pH 2.5 and pH 7.5 for oxytocic activity.

Indices of discrimination

Extracts from five different samples of pooled *ram* semen were assayed, in parallel, on the virgin rat uterus and the rabbit ileum against Pitocin, oxytocic substance (O.S.) and kinin (20). In similar manner, extracts of four more different samples of pooled *ram* semen were assayed on the virgin rat uterus and guinea-pig ileum.

Hydrolysis with enzymes

(a) *Human semen extracts.* The enzymes used were trypsin, chymotrypsin, papain, amino-peptidase and pepsin. To each 2.0 ml. sample of extract was added 6 mg. of one of the above enzymes, and for pepsin-treated extracts the pH was adjusted to 2.5 whereas for the others it was 7.5. These together with control aliquots of extract were incubated at 37° C for 5 to 6 hours. Three control aliquots were also incubated at pH 2.5 and 37° C for 5 to 6 hours.

(b) *Ram semen extracts.* Enzymes as in (a) above were used and also carboxypeptidase, of which 0.1 ml. was added to each 1.5 ml. aliquot of extract. All extracts with

pepsin were assayed on the rat uterus at both pH 2.5 and 7.5 after incubation at 37° C for 5 hours at pH 2.5.

Drugs

All drug doses given in the text refer to the salts. These were atropine sulphate, histamine acid phosphate, mepyramine maleate, acetylcholine chloride, 5-hydroxytryptamine creatinine phosphate, adrenaline hydrochloride and Dibenzyline hydrochloride.

RESULTS

Stability to heat at various pH

Table 1 shows that *ram* semen extracts were largely inactivated by both strong acid and alkali, both at room temperature and on boiling, and that boiling at pH 7.5 caused very little destruction, even after 60 minutes.

TABLE 1. STABILITY OF EXTRACTS OF RAM SEMEN TO HEAT AT VARIOUS pH

Preparation	Percentage inactivation by*							
	pH 7.5 100° C 7.5 min.	pH 7.5 100° C 60 min.	pH 0.4 20° C 7.5 min.	pH 0.4 100° C 7.5 min.	pH 11.5 20° C 7.5 min.	pH 11.5 100° C 7.5 min.	pH 2.5 20° C 60 min. (assayed at pH 2.5)	pH 2.5 20° C 60 min.
1	0	66.6	66.8	80	92.8	>95.6	33.6	76
2	0	0	>96	>96	85	≧85	87.3	55.6
3	0	0	>79	>80.5	≦25	25	75	>75
4	0	0	>87.5	—	5	37.5	>62.5	>62.5

* All extracts assayed at pH 7.5 unless otherwise stated.
> = greater than.
< = less than.
≧ = greater than or equal to.
≦ = less than or equal to.

The table also shows that significant inactivation of these extracts occurred at pH 2.5 and room temperature, and that on testing at pH 7.5 there was no re-activation of the extract.

Hydrolysis with enzymes

(a) *Human semen extracts.* The effects of the enzymes are revealed in Table 2. Amino-peptidase at pH 7.5 and 37° C destroyed 50% or more of the extract's activity in 5 hours. The extracts which were treated with pepsin were tested at pH 2.5, with an apparent loss of at least 66% of their activity, and at pH 7.5, with a loss of 43% of activity. There was little or no destruction of the controls incubated at pH 2.5 and 7.5 without the addition of enzymes.

(b) *Ram semen extracts.* The results of the enzyme experiments are shown in Table 3. Considerable inactivation (more than 63%) of the extracts was found with pepsin when assayed at pH 2.5 but not when tested at pH 7.5. Reference to the stability experiments (Table 1), however, shows that extracts are largely inactivated at pH 2.5 when incubated for 1 hour without enzymes. Table 3 also shows that there was virtually no destruc-

TABLE 2. PERCENTAGE INACTIVATION OF EXTRACTS OF HUMAN SEMEN BY ENZYMES*

Experiment No.	Incubated at pH 2.5			Incubated at pH 7.5				
	Pepsin (assayed at pH 2.5)	Pepsin	Control	Trypsin	Chymotrypsin	Papain	Aminopeptidase	Control
1	>72	—		0	25	0	0	0
2	>59.3	—		0	39.3	0	40.7	0
3	<65	—		—	<68	—	≈47	0
4	28.4	—		9.1	14	9.1	30	30
5	78	58		25	22	30	>78	0
6	>78	—		10	20	0	74	0
7	96.6	63.3		—	—	—	53.3	0
8	>81	63.6		35.6	4	0	75.8	0
9	48	28	0	—	—	—	—	—
10	>50	12.5	25	—	—	—	—	—
11	76.8	>32	0	—	—	—	—	—

* All extracts assayed at pH 7.5 unless otherwise stated.
> = greater than.
< = less than.
≈ = approximately equal to.

tion of the oxytocic material by trypsin, chymotrypsin, carboxypeptidase and papain, and that a variable result was obtained with amino-peptidase.

Indices of discrimination

The indices of discrimination for *ram* semen extract against each of Pitocin, oxytocic substance (O.S.) and kinin are shown in Tables 4 and 5; each active substance was assayed first on the rabbit ileum and rat uterus and then on the guinea-pig ileum and rat uterus. In every case, the effect of the extract could not be abolished by the use of antagonists to histamine (mepyramine), acetylcholine (atropine) or 5-hydroxytryptamine (Dibenzyline). The value of any given pair of tests for discriminating between two active substances can be expressed in terms of the "index of discrimination" (21). The index is equal to the ratio of two results which would be ob-

TABLE 3. PERCENTAGE INACTIVATION OF EXTRACTS OF RAM SEMEN BY ENZYMES*

Experiment No.	Incubated at pH 2.5		Incubated at pH 7.5					Remarks
	Pepsin (assayed at pH 2.5)	Pepsin (assayed at pH 7.5)	Trypsin	Chymotrypsin	Carboxypeptidase	Aminopeptidase	Papain	
1	>78	0	8.3	0	8.3	8.3	11.3	
2	>73.2	—	0	0	0	5.3	0	
3	>75	—	0	0	12	66	0	
4	>75 >60.5	18.7 27.5	0	0	0	6.5	0	
5	>58 >60	0 0	0	0	0	0	0	20 hours incubation
6	50 40	0 0	0	0	0	33	0	20 hours incubation

* All extracts assayed at pH 7.5 unless otherwise stated.
> = greater than.

TABLE 4. INDICES OF DISCRIMINATION: $\frac{\text{RAT UTERUS}}{\text{RABBIT ILEUM}}$

Experiment No.	Semen extract*	Semen extract	Semen extract
	Pitocin	Oxytocic substance	Kinin
1	1.01	0.3	35.5
2	9.3	0.077	11.4
3	0.63	0.112	10
4	1.5	0.6	40
5	2.8	0.05	<12.5
Mean	3.05	0.228	<21.9

* All extracts from *ram* semen.
< =less than.

tained if a standard preparation of one substance were used to assay an unknown extract containing the other. As Gaddum states: "When the index is equal to unity the two tests make no distinction between the two active principles and the results suggest that they may be identical." There was no instance, in our tests, of an index equal to unity.

DISCUSSION

It is assumed from these results that the uterine-stimulating substance found in acid-alcohol extracts of *ram* semen is not oxytocin because (a) it is not inactivated by thioglycollic acid (16 and 17); (b) it is retained during dialysis by a cellophane membrane through which oxytocin passes (1); (c) it stimulates the guinea-pig and rabbit ilea which are relatively insensitive to oxytocin (15); and (d) it is not destroyed by chymotrypsin or papain, both of which inactivate oxytocin (15 and 21).

From the assays on the rat uterus and guinea-pig and rabbit ilea it is evident that the oxytocic principle in extracts of *ram* semen is not identifiable with acetylcholine, HT, histamine, K^+, or adrenaline (19).

The index of discrimination experiments suggest that the unknown substance in our *ram* semen extracts is not oxytocin, or kinin (bradykinin, P.P.S., kallidin, substance U or substance Z) (22), or oxytocic substance

TABLE 5. INDICES OF DISCRIMINATION: $\frac{\text{RAT UTERUS}}{\text{GUINEA-PIG ILEUM}}$

Experiment No.	Semen extract*	Semen extract	Semen extract
	Pitocin	Oxytocic substance	Kinin
1	52	0.5	< 3.43
2	> 10	0.43	6.31
3	>354	0.67	34.5
4	>890	<0.37	<116.6
Mean	>326.5	<0.49	< 40.2

* All extracts from *ram* semen.
< =less than.
> =greater than.

(O.S.) of our blood extracts (1, 2, 3, 4, 5, 6 and 23). This view is supported by the enzyme hydrolysis results which showed that oxytocic substance of our blood extracts is destroyed by chymotrypsin and carboxy-peptidase, whilst kinin is inactivated by both these enzymes and also by trypsin and papain (15). None of these enzymes has any significant effect on the active substance in *ram* semen extracts (or in *human* semen extracts).

The uterine-stimulating substance in extracts of *human* semen is also quite distinct from oxytocin, K^+, acetylcholine, histamine, HT, adrenaline, kinin and blood oxytocic substance, on the basis of the results obtained.

From the enzyme experiments, it was observed that the active substance in extracts of *human* semen differed from that in *ram* semen extracts in being partially destroyed by amino-peptidase, and in being unaffected by exposure to pH 2.5 for more than an hour at 37° C.

Acidifying *ram* semen extracts to pH 2.5 for 1 hour resulted in considerable loss (more than 65%) of oxytocic activity when tested on the rat uterus at pH 2.5. On adjusting the pH from 2.5 to 7.5 the loss of activity was of the same order. A curious observation was that on acidifying to pH 2.5 and incubating with pepsin at 37° C for 5 hours there was a similar order of inactivation when tested at pH 2.5 on the rat uterus. Virtually no loss of activity occurred however ($<6.6\%$) when the pH was corrected to 7.5 after incubating with pepsin at pH 2.5 and 37° C for 5 hours. This suggests that acidification, and not pepsin, causes the inactivation of the active substance in the extracts, and that during neutralisation of the pepsin-treated extracts re-activation of the oxytocic substance occurs due to pepsin (or another uterine-stimulating factor is perhaps liberated by pepsin activity) (24).

Chymotrypsin is reported to destroy vasopressin, pepsanurin, pepsitocin, substance P, hypertensin (angiotonin or angiotensin) and pepsitensin; trypsin has been found to inactivate vasopressin, pepsanurin, substance P, hypertensin and pepsitensin; and pepsin is known to destroy substance P, hypertensin and pepsitensin (21). On the basis of this documented evidence the active uterine-stimulating substance in our extracts of *human* and *ram* semen is not considered to be vasopressin, pepsanurin, substance P, hypertensin or pepsitensin.

From our evidence it is doubtful whether the unknown uterine-stimulating substances in extracts of *human* and *ram* seminal fluid are polypeptide in nature, and further investigations are being made to determine whether the active principle in extracts of either *human* or *ram* semen might be allied to the substance known as prostaglandin (10, 25), which has been isolated from the seminal fluid of sheep, man and goats. Prostaglandin is a nitrogen-free carboxylic acid, probably unsaturated and with hydroxy groups (10).

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INFLUENCE OF AGE, SEX AND ADRENOCORTICAL STATUS ON HEPATIC REDUCTION OF CORTISONE *IN VITRO*[1]

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ABSTRACT

Whole liver homogenates from male and female rats were studied for their ability to reduce the 17,21-dihydroxy-20-ketone sidechain and Δ^4-3 ketone group of cortisone. The previously reported sex difference in both sidechain and ring A reduction was confirmed. Sidechain reduction is more rapid in the male; ring A reduction more rapid in the female. Castration of the male depressed sidechain reduction and increased the rate of ring A metabolism. These changes were prevented by testosterone administration if started immediately following surgery. Ovariectomized female rats showed no change in ring A activity and only a slight increase in the rate of sidechain reduction. Estradiol to males and testosterone to females depressed the sidechain and ring A reduction respectively. Neither pregnancy nor progesterone administration significantly influenced cortisone metabolism in homogenates from female rats. Injections of cortisone acetate reduced C-20-keto reductase activity in both males and females, had no effect on Δ^4-3 ketone reduction in females but sharply depressed this function in males. Young rats from 5 to 75 days of age were studied at various intervals to determine the manner in which the sex difference in cortisone metabolism develops and how this is modified by castration.

NUMEROUS studies have been reported concerning the metabolism of adrenocortical steroids by liver homogenates. The major metabolic changes responsible for inactivation appear to be C-20 and Δ^4-3 ketone reduction (1, 2, 3, 4). Ring A reduction by rat liver enzyme systems gives rise to the corresponding dihydro and tetrahydro derivatives (1) while reduction at the C-20 ketone position produces both the C-20 alcohol with intact ring A and further reduction to the corresponding pentol (4). Reduced triphosphopyridine nucleotide serves as a source of hydrogen in the reduction of both ring A and sidechain (5, 6, 2, 7). The reduced derivatives are subsequently conjugated with glucuronic acid in the liver (8) and removed from the circulation by excretion through the kidney (9). The reductive steps in the metabolism of cortical steroids represents inactivation insofar as the dihydro and tetrahydro derivatives and C-20 reduced products (Reichstein's E and the pentol) are inactive in the rat liver glycogen deposition assay (10).

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Work on the problem of enzymatic inactivation of adrenocortical hormones has developed along two distinct but complementary lines, one to study the intracellular localization and mechanism of the reactions involved and the other to establish the nature of the reaction products. Only recently has a third major approach been instituted with the investigation of various external and internal environmental factors which influence the hepatic capacity to metabolize steroids. In this respect, gonadal hormones have been shown to play a significant role. Liver homogenates from adult male rats reduce the 17,21-dihydroxy-20-ketone sidechain of cortisone at a more rapid rate than do similar preparations from female rats (11, 7). The female rat, on the other hand, metabolizes the Δ^4-3 ketone group faster than does the male (12, 13, 7). In previous studies (7) designed to elucidate the basis for the sex difference in sidechain metabolism, it was found that gonadectomy decreased the rate of cortisone reduction in the male and increased the rate in females. Estradiol decreased C-20-keto reductase activity in males while testosterone increased this activity in the female. Much of this work was done before the critical importance of TPNH in the *in vitro* system was appreciated. This is taken into account in the present report which extends earlier studies on the manner in which sex, age and endocrine status affects hepatic enzyme systems responsible for reduction of cortisone.

METHODS

Rats, from the Holtzman strain, were studied when 100 to 150 days of age except when otherwise stated. They were killed by a blow on the head followed by section of the cervical vessels. The liver was rapidly excised, chilled and weighed. Homogenization was performed at 0° C in 0.25 M sucrose which contained 0.04 M nicotinamide. Four ml. aliquots of whole homogenate containing 200 mg. of tissue were incubated with 150 μg. of cortisone. To each incubation vessel was also added 0.01 ml. of 0.3 M manganese chloride, 0.1 ml. of 0.2 M sodium isocitrate and 0.4 ml. of 10^{-4} M TPN. The addition of isocitric dehydrogenase was found, in earlier experiments (6, 7), to be unnecessary for maximal TPN effect when whole homogenates were used and therefore was not added. Potassium phosphate buffer maintained pH at 7.4 and incubation was for 2 hours at 37° C in an atmosphere of nitrogen.

Following incubation, the extraction procedure was exactly as described previously (7). Reduction of the sidechain was determined by the Porter-Silber reaction (14) and loss of optical density at 240 mμ. was the measure of ring A reduction.

All surgical procedures were carried out under ether anesthesia. Adrenalectomized animals were maintained on dry sodium chloride *ad libitum*. This method of administering salt has the advantage over saline drinking water of allowing the animal a finer control over his salt intake as dictated by his requirements. In our laboratory, this has consistently allowed weight gains in young adrenalectomized rats.

RESULTS

Effects of gonadal hormones on reduction of cortisone

That liver homogenates from male and female rats reduce the sidechain and ring A of cortisone to different degrees is clear from the data in Table

TABLE 1. THE EFFECT OF VARIOUS FACTORS ON REDUCTION OF THE 17-21-DIHYDROXY-20-KETONE SIDECHAIN AND Δ^4-3 KETONE GROUP OF CORTISONE BY LIVER HOMOGENATES FROM ADULT MALE AND FEMALE RATS

Group No.		No. of rats	Micrograms cortisone reduced	
			Sidechain[1]	Ring A[2]
	Males			
1.	Normal males	13	113 ±3.5	75 ±2.5
2.	Castrated males, 18 days post-op.	11	85 ±4.6	104 ±5.3
3.	Castrated males, testosterone started 10 days post-op.; given for 10 days. 2.5 mg./d. I.M.	4	80 ±7.5	80 ±9.6
4.	Castrated males, testosterone propionate started immediately post-op.; given for 12 days. 2.5 mg./d. I.M.	10	101 ±4.5	81 ±3.0
5.	Intact males, estradiol 60 μg./d./10d. S.Q.	8	63 ±7.2	78 ±6.0
6.	Males, ACTH 2 units/d./10d. S.Q.	6	112 ±6.0	80 ±3.1
7.	Males, cortisone acetate 5 mg./d./10d. I.M.	6	67 ±8.3	48 ±3.9
8.	Adrenalectomized males, 10 days post-op.	7	90 ±4.6	89 ±3.3
	Females			
9.	Normal females	18	53 ±3.8	113 ±1.6
10.	Ovariectomized females, 40 days post-op.	7	69 ±7.1	109 ±3.7
11.	Intact females, testosterone propionate 2.5 mg./d./10d. I.M.	6	61 ±5.8	63 ±6.3
12.	Pregnant females	7	62 ±6.9	110 ±2.3
13.	Females, progesterone 2.5 mg./d./10d. S.Q.	6	54 ±8.6	115 ±6.9
14.	Females, cortisone acetate 5 mg./d./10d. I.M.	6	23 ±9.3	110 ±7.8
15.	Adrenalectomized females, 10 days post-op.	6	59 ±3.4	82 ±1.5

[1] Porter-Silber reaction.
[2] Ultra-violet absorption at 240 mμ.
Results are expressed as means ±S.E.

1. The male reduces the sidechain more rapidly than does the female who, in turn, reduces ring A at a faster rate than does the male. Castration of the adult male 18 days prior to removal of the liver (Group 2, Table 1) produces changes toward the female pattern, i.e., sidechain metabolism is moderately reduced and ring A reduction is markedly increased. In view of this, it was reasoned that testosterone replacement in castrated males might reverse the observed effects. In Group 3 of Table 1, testosterone (aqueous suspension) was started 10 days following castration and given for a period of 10 days at the rate of 2.5 mg. per day I.M. Ring A changes were reversed but sidechain metabolism remained depressed. The animals in Group 4 were castrated and immediately started on testosterone propionate in oil (2.5 mg./day, I.M.) which was continued for 12 days before the livers were studied. Again it is clear that the ring A values return to normal and, in this case, there was also a significant (Group 4 compared to Group 2: $P = <.02$) increase in sidechain reduction which approached that for the intact male. This seems to indicate that once androgenic support for C-20-keto reductase has been absent for several days, testosterone will not re-establish the normal activity but if replacement is begun immediately following surgery the activity is sustained to a good degree. The fact that free testosterone was used in Group 3 and the more pro-

longed acting testosterone propionate in Group 4 might also have some bearing on the results.

The male animals in Group 5 were injected, subcutaneously, with estradiol (aqueous suspension) for 10 days, 60 μg. per day. Sidechain reduction is suppressed to the female level but ring A metabolism is not different from untreated male rats. Since reduction of sidechain activity is significantly greater than that produced by castration (Group 5 compared with Group 2: P = <.02), it is probable that estradiol exerts its effect by a mechanism other than depression of testicular function although this doubtlessly occurs to some degree with the dose of estradiol used. This contention is further supported by earlier studies (7) in which the decrease in sidechain activity in castrated males was even more marked following estradiol administration to the operated animals. The decrease in sidechain activity following castration may then be viewed as being due to removal of supporting androgens while the depression following estradiol would be due to the presence of inhibiting or competing estrogen.

The situation must, however, be different in the case of ring A metabolism. Here testosterone acts to maintain rate of reduction at the relatively low male level while estradiol is without effect (Group 5). A comparable mechanism appears to operate in the female. Intact females and ovariectomized females (Groups 9 and 10) have the same ring A reducing activity, indicating that estrogen plays no part here. Testosterone propionate does, however, produce a striking reduction of ring A metabolism (Group 11) and this is the most profound change elicited by gonadal hormones in the female with respect to either the ring A or sidechain. Ovariectomy and testosterone administration (Groups 10 and 11) both produced an apparent increase in C-20-keto reductase activity which, however, was not impressive. In the earlier report (7) testosterone and ovariectomy produced very significant increases in the rate of sidechain reduction in the female. The two experiments differ only in that reduced TPN was present in the current study but not in the earlier one.

Development of sex difference in rate of sidechain reduction and the effect of castration

Figure 1 depicts the ability of liver homogenates from young male and female rats (5 to 75 days old) to reduce the sidechain of cortisone. Male animals 5 to 15 days old had significantly (P = <.001) less activity than females at this age. This difference is not seen in the 15 to 25 day group and from this point there is no significant sex difference until about the 55th day of life. At this time the sex difference characteristic of the adult becomes established. Female C-20-keto reductase activity remained essentially unchanged throughout the 5 to 75 day interval studied with the possible exception of a slight fall in the 55 to 75 day group. This value was, however, not statistically different (P = >.05) from the 35 to 55 day

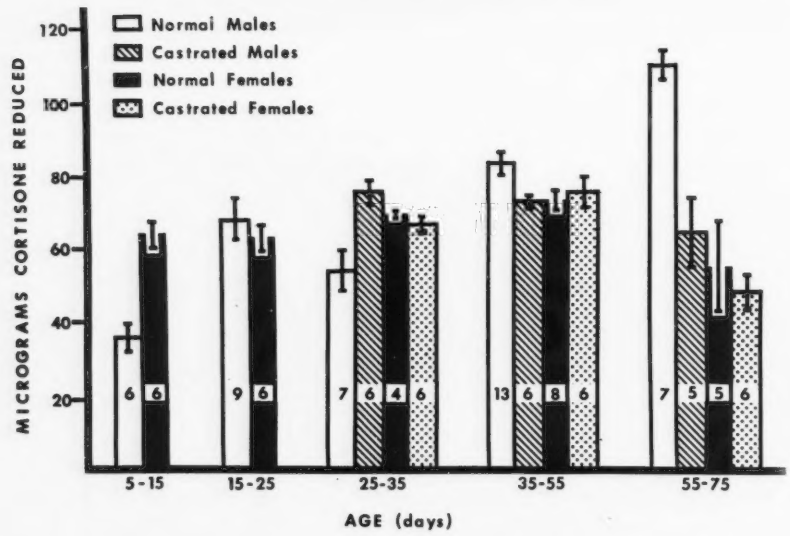

FIG. 1. Rate of 17,21-dihydroxy-20-ketone reduction as influenced by age, sex and castration. Castration was performed when the animals were 15 days of age and cortisone reducing activity of the livers was assayed at the ages indicated. Each bar represents the mean value for the number of animals indicated in the open space. Standard errors of the mean for each group are shown.

group. The castrated animals were operated at 15 days of age and assayed at the times shown. Values for the ovariectomized females were not different from those of the intact females at any age studied. Castrated male rats, studied when 30 days old, reduced the sidechain at a faster rate than did the intact males of this age group ($P = <.01$). The reason for this is entirely obscure and no difference is noted between the operated and unoperated males in the 35 to 55 day group. It is clear, however, that castration prevents the male from reaching the normal adult value at 55 days of age; activity instead remains in the female range.

Development of sex difference in rate of ring A reduction and the effect of castration

In Figure 2 it is seen that the rate of ring A reduction in the young male progresses to a peak value at about 30 days of age and is maintained through age 55 days, at which time it drops to the adult value. Castration modifies the development of this function in that the sharp rise at 25 to 35 days of age is prevented and a very significant ($P = <.001$) rise over the intact male value is made possible in the 55 to 75 day group. Female activity follows closely that of the male for approximately the first 55 days of life, there being no significant sex difference during this period. Shortly thereafter, however, an abrupt increase in female activity is ob-

served (55 to 75 day group) at the time male function is decreased, resulting in the adult sex difference in ring A reducing activity. Castration at age 15 days renders the female activity noticeably lower at both the 25 to 35 and 35 to 55 day intervals but exerts no influence on the 55 to 75 day group. In the former two groups, the effect might be explained on the basis of an overall, non-specific decrease in metabolic activity due to absence of the gonads. This is supported by the observation that gonadectomized males also exhibit this reduction in enzyme activity. Be this as it may, an entirely different mechanism is indicated in the case of the operated animals 55 to 75 days of age. Here the loss of ovarian function has no influence while loss of testicular secretion allows the male to approach the female level of enzyme activity.

Pregnancy and progesterone administration

There is much evidence that a considerable increase in adrenal cortical activity occurs during pregnancy (15, 16, 17, 18). Possible changes in the inactivation of adrenocortical hormones during pregnancy have, however, received little study. Accordingly, the ability of pregnant rats to reduce cortisone was studied. Each animal carried from 10 to 15 fetuses with an average nose to rump length of 25 mm. The data in Group 12 of Table 1 indicate a slight increase in rate of sidechain reduction which, however, is

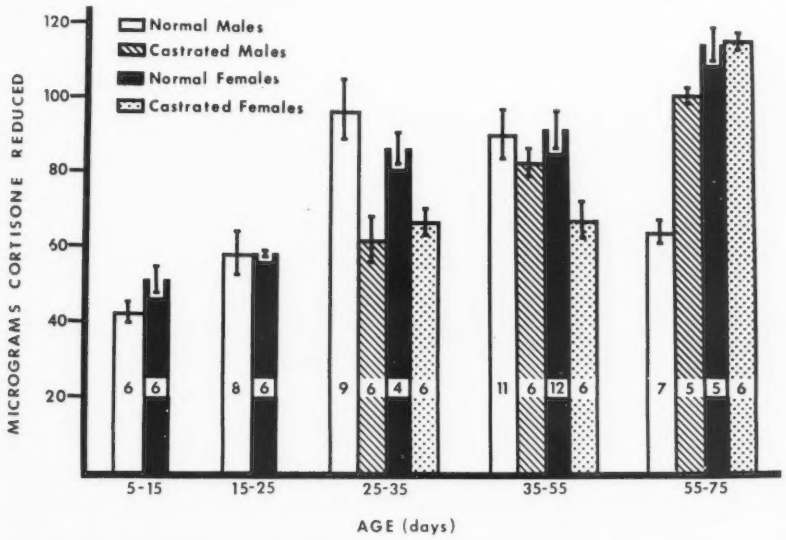

FIG. 2. Rate of Δ^4-3 ketone reduction as influenced by age, sex and castration. Castration was performed when the animals were 15 days of age and cortisone reducing activity of the livers was determined at the ages indicated. Each bar represents the mean value for the number of animals indicated in the open space. Standard errors of the mean for each group are shown.

not statistically significant (P= >.05). No effect on ring A is evident. In view of the tendency toward an increase in sidechain reduction during pregnancy, it was of interest to investigate the influence of progesterone on the enzyme systems responsible for cortisone inactivation. It is clear (Group 13) that progesterone administration did not modify cortisone reduction.

Effects of hypercorticalism and adrenalectomy in adult males and females

ACTH, in aqueous suspension, was given to male rats for 10 days, 2 units per day. This produced a 20% increase in adrenal weight (ACTH-treated: 15.2 ± .67 mg./100 gm. Control: 12.6 ± .03 mg./100 gm.) but no effect on hepatic reduction of cortisone (Group 6). Cortisone acetate, administered 5 mg. per day for 10 days, did, however, produce changes in both the male and female. In the male (Group 7), C-20-keto reductase activity as well as reductive activity on ring A were sharply reduced and to about the same degree. In the female, on the other hand (Group 14), sidechain reduction was depressed about 50% with no apparent effect on ring A metabolism. This dichotomy of effect argues against the possibility that the observed responses are simply non-specific manifestations of the protein catabolic action of cortisone. Rather it would appear that a specific substrate suppression of the enzymes has occurred.

Another series of male rats were treated with cortisone acetate, 5 mg. per day, for various periods of time. Figure 3 shows that no effect was observed until after three days of injections at which time both sidechain and ring A reduction were increasingly depressed, reaching a maximum at about 9 days. After this point, both enzyme systems tend to adjust to the high concentration of substrate. However, only the ring A system appears to compensate completely during the 15 days of treatment.

The adrenalectomized animals of Groups 8 and 15 were operated 10 days before their livers were removed for study. The response was again sex dependent. Males exhibit a decreased C-20-keto reductase activity and an increased rate of ring A reduction (P= <.001). The females show a slight increase in ability to reduce C-20, which is not statistically significant, and a very significant decrease in ring A reducing activity.

DISCUSSION

There is good evidence that the liver plays a significant role in the regulation of both the degree and duration of the biological response to a steroid hormone (10). It thus becomes of great importance to ascertain the factors which influence this hepatic mechanism. The thyroid has been shown to influence hepatic reduction of corticosteroids (19, 20) and the practical significance of this information has been demonstrated in at least one clinical study (21). Insofar as judgments can be made from *in vitro* studies, some of the relationships demonstrated in the present report

might reasonably be expected to have functional significance in the disposition of adrenocortical hormones in the intact animal. In this category are the findings that sidechain reduction is supported and ring A reduction is depressed by testosterone in the male. The adult female, while reducing the ring A much more rapidly than the sidechain, has no appreciable gonadal regulation of these reactions. The fact that high levels of testosterone depress ring A reduction in the female (Group 11) might, however, become important in situations in which there is an increased output of adrenal androgens.

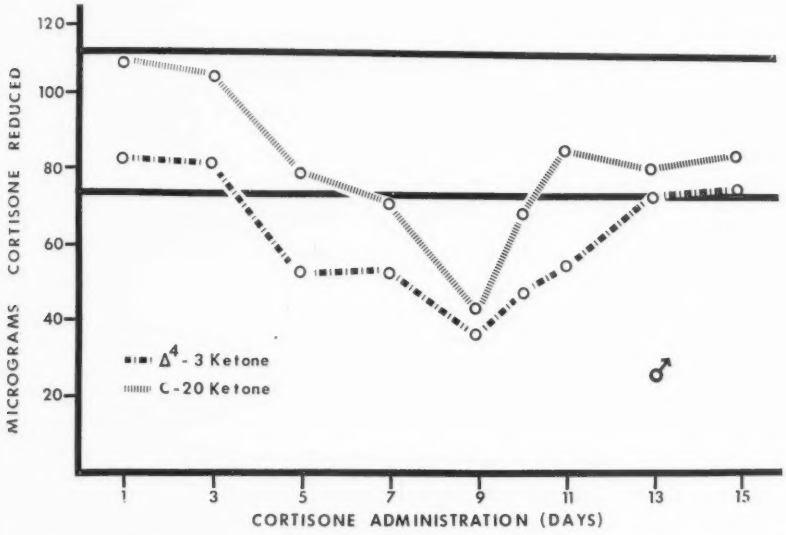

FIG. 3. Effect of *in vivo* cortisone acetate administration (5 mg. per day) on hepatic reduction of cortisone *in vitro*. Each point represents the mean value for two male rats. The solid horizontal lines indicate mean values for C-20 ketone and Δ^4-3 ketone reduction by non-treated males.

Supporting evidence for the operation of these mechanisms in the intact organism is meager but not entirely absent. Abelson and Borcherds (22) report that human females remove injected cortisol from the blood at a slower rate than do males. Estrogen administration has been shown to diminish the rate of cortisol reduction in human patients (23).

If it proves to be correct that the male and female metabolize steroid hormones in a characteristically different manner, the question arises as to the utility of this arrangement to the animal. Separate pathways for synthesizing more physiologically active or physiologically different steroids may be involved in the two sexes. The oxidation and reductions of cortisone and its various products may be directly associated with the mechanism of hormone action. Indeed, a coenzymatic action of steroids

has been demonstrated (24) and it is postulated that the action and metabolism of steroid hormones may be part of the same process. The kind of cell acted on and its environment may direct the reductive process and the resulting products.

Studies (25, 26, 27) relating to the effect of non-specific stress on the rate of metabolism of adrenocortical steroids have, in general, shown a decrease under these conditions. Normal or decreased metabolism of intravenously administered cortisol has been observed in man following the stress of a surgical operation (28). We have shown that a sustained state of hypercorticalism, produced by cortisone injection, will decrease cortisone sidechain reduction by both male and female rats. It may be postulated that the increased adrenocortical secretion, which occurs in stress, promotes a decreased rate of cortical steroid inactivation. This would allow a higher and more prolonged blood level of corticoids at less cost to the organism.

We are unaware of other studies which parallel those reported here on sidechain metabolism. Yates and coworkers (12, 29) have, however, conducted comprehensive investigations on ring A reduction of several corticoids by liver homogenates under various experimental conditions. Although interpretation of the data may differ, there is good agreement between their findings and those in this report. Both studies demonstrate that: 1) homogenates of liver from adult female rats reduce ring A of Δ^4-3-keto steroids at a faster rate than do those from males; 2) castration increases and testosterone decreases ring A reduction in males; 3) in adult females, castration has no appreciable effect; 4) testosterone causes a marked decrease of activity in adult females; 5) females, castrated when very young and allowed to grow to adulthood, develop the high level of activity characteristic of the female. Males, studied in the same manner, reach a level of activity much higher than the intact male; a level which in our study did not differ from the adult intact or castrated female (Fig. 2); 6) in males, treatment with cortisone causes a loss of activity which, according to our study, is time or dose dependent and will return to normal if cortisone is given for as long as 12 days (Fig. 3); 7) substrate depression of hepatic activity is more difficult to demonstrate in the female.

In considering the points of disagreement between the two studies, it must be emphasized that comparisons can be drawn only on the ring A findings since Yates' group did not study the sidechain. The most apparent differences are: 1) estradiol administration to adult males produced no change in hepatic activity in our study but increased activity in the other investigation. The doses were 60 μg. per day for 10 days and 1 μg. per day for 14 days respectively. It is difficult to explain the discrepancy since lack of effect is obviously not due to insufficient estrogen administration; 2) ACTH, 2 units per day for 10 days, to adult male rats produced no change but 10 units per day for 12 days (29) caused a 30% depression.

This could be a dosage difference; 3) male rats, adrenalectomized and maintained on dry sodium chloride showed a significant increase in activity while Yates *et al.* (12) report no change when saline drinking water was used.

Acknowledgments

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THE EFFECT OF TRYPAN BLUE ON THYROID FUNCTION IN THE RAT[1]

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ABSTRACT

Four ml. of a 0.5% saline solution of trypan blue were injected intraperitoneally once or twice daily for varying periods of time. Six days after the injections, thyroid weight and serum PBI had decreased to 60% and 25% of normal, respectively, while thyroidal radioiodine uptake was suppressed to 15% of the normal value. Thyroid hormone secretion was inhibited for about 14 hours by a single injection of trypan blue; continuous inhibition was produced by repeated injections. Exogenous TSH given to these treated rats caused thyroid hormone secretion to resume.

Adrenal weight increased after trypan blue administration; but there was no change in testis weight. Pituitary weight was slightly less in treated animals.

It is believed that trypan blue treatment specifically inhibits pituitary TSH secretion, causing secondary hypofunction of the thyroid.

A NUMBER of studies have shown that some drugs and dyes bind with serum proteins and that such bindings may be responsible for the biological activity of the drugs (1, 2, 3). Binding to serum proteins has also been demonstrated with several hormones, namely, thyroxine (4, 5), adrenocortical hormone (6), posterior pituitary hormone (7), and estrogen (8). Several clinical and experimental studies have shown that thyroid status does not always correlate with the level of circulating protein-bound thyroxine (9, 10, 11). It has been postulated that the biological action of thyroxine depends on the concentration of circulating "free" thyroxine rather than on that of the protein-bound hormone (4). Recently it has been shown that trypan blue competes with thyroxine for binding sites on serum protein *in vitro*, thus presumably making more free thyroxine available to the cells (12). It was therefore considered of interest to study the effect of trypan blue on the pituitary-thyroid system, since the increased thyroxine theoretically produced by trypan blue treatment would be expected to suppress thyrotropin secretion by the pituitary.

MATERIALS AND METHODS

One hundred and fifteen adult male Holtzman rats, weighing 158 to 239 gm., were employed. To permit an adequate radioiodine uptake, the animals were fed a Remington low iodine diet beginning seven days before the experiment. Body weight was measured

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t the same time daily and the diet of the control animals was regulated to keep their eight similar to those of the trypan blue-treated rats. A 0.5% solution of trypan blue in hysiological saline was injected intraperitoneally as follows: Group A, 4 ml. once daily r three days; Group B, 4 ml. twice daily for three days; Group C, 4 ml. once daily for x days; Group D, 4 ml. twice daily for six days. I^{131} (0.2 μC.) was injected subcutaneusly six hours before autopsy, which was performed the day after the final injections trypan blue.

At autopsy, pituitaries, thyroids, adrenals and testes were cleanly dissected and eighed. The thyroid radioiodine uptake was measured in a well-type scintillation unter. Organ weight/body weight ratio was calculated from the initial body weight d expressed as a percent of that of the control animals.

Blood was taken from the animals of the control, C and D groups, and was then oled in each group. Serum concentration of total iodine and protein-bound iodine BI) were determined by the Bio-Science Laboratory, Los Angeles, California.

The animals in which thyroid hormone secretion was measured first received 20 μC. of I^{131} intraperitoneally. Beginning twenty-four hours later, 30 mg. of propylthiouracil (PTU) in 10% gum acacia were injected daily subcutaneously at rotated sites to block the reaccumulation in the thyroid of I^{131} liberated from the degradation of endogenously labeled iodinated amino acids. Counts over the thyroid region were made three to five times daily under light ether anesthesia with an externally placed scintillation counter. Each point was expressed as a percent of the initial thyroid uptake on semi-log paper. When a straight line of more than five individual points had been obtained, the trypan blue injections were given, either intravenously or intraperitoneally. Six animals received a single injection of 4 ml. of trypan blue. Three animals received three injections of 4 ml. of trypan blue at 12-hour intervals, and six hours after the last injection received 0.68 U.S.P. units of thyrotropin intraperitoneally.

In another 31 animals, beginning 24 hours after I^{131} injection, 20 μg. of l-thyroxine were injected intraperitoneally daily, in addition to the PTU. After thyroidal secretion had been measured for 65 hours, 4 ml. of the trypan blue solution were injected intraperitoneally. Three hours later graded doses of TSH (0.002, 0.02 and 0.2 U.S.P. units) were injected subcutaneously.

RESULTS

Preliminary experiment

An initial attempt was made to discover which solvent, water or saline, and which type of injection, intravenous or intraperitoneal, would be more satisfactory, giving 4 ml. of a 0.5% solution once daily for three days. The results of this study are shown in Table 1. The method of injection had no effect on thyroid weight, thyroidal radioiodine uptake, adrenal weight or testis weight. It was also apparent that the nature of the solvent was of no consequence. It was decided to use a saline solution of trypan blue

TABLE 1. EFFECT OF TRYPAN BLUE ADMINISTRATION ON THYROID, ADRENAL AND TESTIS WEIGHT AND THYROIDAL I^{131} UPTAKE

Group	Number of animals	Initial body wt., gm.	Thyroid wt., mg./100 gm.	Adrenal wt., mg./100 gm.	Testis wt., gm./100 gm.	Thyroidal I^{131} uptake, %
Intravenous T.B.	4	174 (±6)	7.79 (±1.13)	25.16 (±0.94)	1.51 (±0.04)	7.6 (±1.3)
Intraperitoneal T.B.	4	173 (±5)	8.09 (±0.92)	25.81 (±1.33)	1.61 (±0.09)	7.6 (±1.2)
Control	4	168 (±4)	10.02 (±0.41)	18.30 (±1.18)	1.38 (±0.05)	14.3 (±1.2)

Results expressed as mean ± standard error of mean. Two animals of each group received a saline trypan blue (T.B.) solution; the remaining two received aqueous T.B. No difference was apparent between the two solvents.

and to administer the drug intraperitoneally in the remaining experiments.

The possible toxicity of trypan blue was investigated by giving 4 ml. twice daily for 10 days. The results of this experiment are shown in Table 2. None of the animals died during the study. A pronounced suppression was noted in the thyroidal radioiodine uptake of the trypan blue-treated rats, and the thyroid glands of these animals were markedly smaller than those of the saline injected controls. However, no attempt was made in this experiment to regulate the body weight of the control animals. The

TABLE 2. EFFECT OF CHRONIC TRYPAN BLUE ADMINISTRATION ON THYROIDAL RADIOIODINE UPTAKE, THYROID, ADRENAL, TESTIS AND BODY WEIGHT

Group	Number of animals	Initial body wt., gm.	Final body wt., gm.	Thyroid wt., mg./100 gm.	Adrenal wt., mg./100 gm.	Testis wt., gm./100 gm.	Thyroidal I^{131} uptake, %
TrypanBlue	7	187 (±2)	152 (±3)	5.38 (±0.20)	34.80 (±0.61)	1.54 (±0.10)	6.05 (±1.38)
Saline Controls	6	189 (±3)	206 (±3)	8.72 (±0.24)	20.51 (±0.44)	1.44 (±0.05)	39.70 (±4.90)

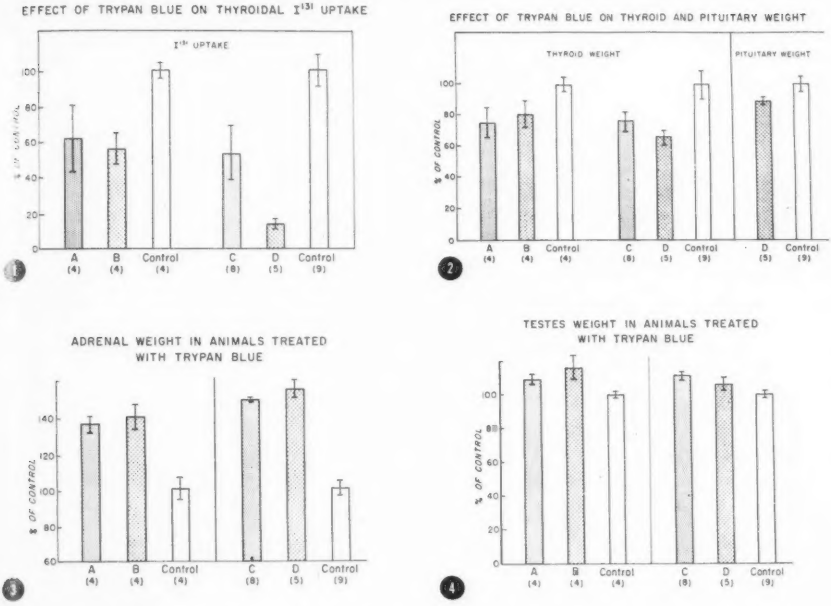
Results expressed as mean ± standard error of mean. The control group received 4 ml. of saline intraperitoneally

final body weight/initial body weight ratio was 0.8 in the trypan blue group, while it was 1.1 in the saline group. Since this difference in body weight probably reflected variation in the amount of low iodine diet ingested, and this in itself might influence thyroid size and radioiodine uptake, the diet was carefully controlled in the remaining experiments.

The effect of trypan blue on thyroid function

The effect of trypan blue administration on thyroidal radioiodine uptake is shown in Figure 1. The radioiodine uptake was suppressed in all trypan blue-treated groups. Although there was no significant difference in the uptake of the two groups (A and B) treated for three days, in the groups treated for six days there was a marked difference between those animals injected once a day (C) and those injected twice a day (D). The uptake in Group D was only 14% of the control level, while in Group C it was not different from that in Groups A and B. The concentration of total and protein-bound iodine in the serum was measured only in Groups C and D (Table 3). PBI was less in both the C and D groups but its decrease was surprisingly marked in D, where it was found to be only 25% of the control. Total iodine was similarly low in both groups. Thyroid weight decreased in all trypan blue-treated animals and was smallest in Group D (Fig. 2).

The effect of trypan blue on the release of organically bound radioiodine from the thyroid gland is shown in Table 4. Inhibition was produced in all animals by a single injection of trypan blue. This inhibition appeared promptly after the injection and lasted from 9 to 24 hours (Fig. 5). There was no marked difference between the intravenously injected and intraperitoneally injected groups. It was not possible to measure the thyroidal



FIGS. 1–4. Four ml. of 0.5% saline solution of trypan blue were injected intraperitoneally daily as follows: (A) once daily for 3 days, (B) twice daily for 3 days, (C) once daily for 6 days and (D) twice daily for 6 days. Four ml. saline were injected in the same way in the control group. I^{131} was injected subcutaneously six hours before autopsy. The height of the bar and vertical line represent the mean and standard error of the mean. Figures in parentheses indicate the number of animals in each group.

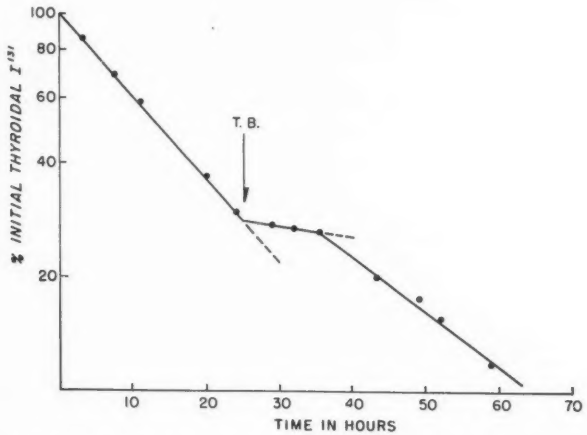


FIG. 5. Effect of intraperitoneal administration of trypan blue on thyroid hormone secretion.

TABLE 3. EFFECT OF TRYPAN BLUE ON TOTAL IODINE AND PBI CONCENTRATION IN THE SERUM

Group	Total iodine		P.B.I.	
	μg./100 ml.	% of control	μg./100 ml.	% of control
Control	2.4	100.0	2.4	100.0
C group	1.5	62.5	1.1	45.8
D group	0.6	25.0	0.6	25.0

Trypan blue was injected intraperitoneally once daily in C and twice daily in D for 6 days. Saline was injected twice daily for 6 days in the control animals. Serum was pooled in each group.

secretion rate accurately during inhibition because the period was so short, but it seemed that complete inhibition was not produced in all animals by a single injection of trypan blue.

Continuous inhibition of hormone release in three animals was produced by repeated trypan blue injections. In one, only a slight change in the thyroidal secretion rate was produced, but quite striking suppression occurred in the other two animals (Fig. 6). After thyrotropin administration, thyroidal secretion increased significantly, indicating that there was no impairment in the ability of the thyroid to respond to TSH.

A comparison of the effect of graded doses of exogenous TSH on thyroidal secretion rate in trypan blue-treated and control animals (Fig. 7) showed essentially no difference in the responses of the two groups at the various dose levels used (0.002 to 0.2 U.S.P. units).

In four control animals, saline alone was injected once or three times (every 10 hours), and thyroidal radioiodine release was studied for 70 hours. None of the animals demonstrated a change of the secretion slope after either single or repeated injections of saline. In 12 untreated control

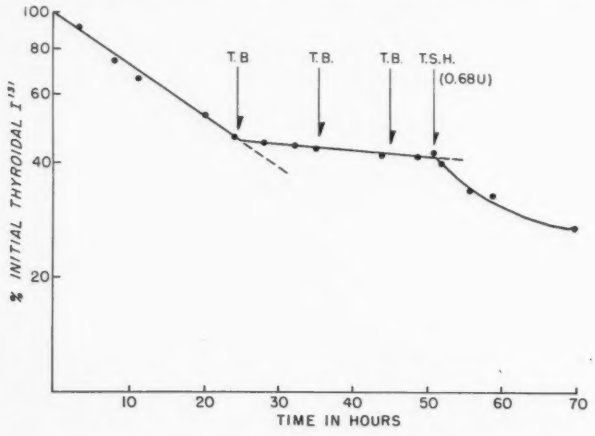


FIG. 6. Effect of repeated injection of trypan blue and subsequent administration of TSH on thyroid hormone secretion.

TABLE 4. EFFECT OF TRYPAN BLUE ON THYROIDAL HORMONE SECRETION

Animal number	Trypan blue‡		Duration of inhibition (hrs.)
	Dose (ml.)	No. of injections	
1	4	1*	10
2	4	1*	10
3	4	1*	24
4	4	1	10
5	4	1	9
6	4	1	19
7	4	3	more than 40
8	4	3†	more than 40
9	4	3†	more than 40

* = intravenous injection.
† TSH was injected intraperitoneally 6 hours after the last injection of trypan blue.
‡ Trypan blue was injected intraperitoneally or intravenously.

animals thyroidal secretion was exponential throughout the period of observation.

The effect of trypan blue on the weight of the adrenals, testes and pituitaries

Adrenal weight increased markedly in all trypan blue-treated animals (Fig. 3). The degree of adrenal enlargement seemed to be related to the dose of trypan blue and to the duration of treatment.

Rather surprisingly, the testes weighed slightly more in the treated than in the control animals, although the difference is of questionable signifi-

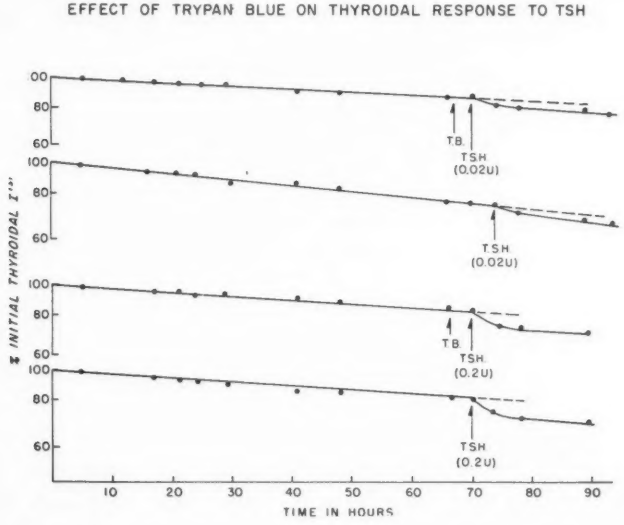


FIG. 7. Thyroidal hormone secretion responded normally to graded doses of TSH in trypan blue-treated animals. Four ml. of 0.5% trypan blue were injected intraperitoneally and 3 hours later graded doses of TSH were injected subcutaneously. Endogenous TSH was inhibited by daily injection of large doses of thyroxine.

cance (Fig. 4). This slight increase in testis size in the treated animals was also noted in the two preliminary experiments (Tables 1 and 2). On the other hand, the pituitary weight of the treated animals was slightly less than that of the controls (Fig. 2).

Miscellaneous findings

The injected trypan blue was excreted primarily in the urine. The red contaminant of trypan blue appeared in the urine before trypan blue itself. All body tissues were stained deep blue except the major part of the brain. In the brain, only the pituitary, pineal body, and choroid plexus were stained blue. The intermediate lobe and that part of the adenohypophysis just adjacent to the intermediate lobe were the most deeply stained.

DISCUSSION

The level of circulating thyroid hormone, as measured by the PBI level, has been thought to regulate thyroid function by controlling the secretion of thyrotropin by the adenohypophysis, thus maintaining a constant level of circulating thyroxine (13). This theory, however, does not fit all clinical and experimental findings. Pregnant women usually have high PBI levels but are clinically euthyroid (14, 15). On the other hand patients with the nephrotic syndrome frequently have low PBI levels but again are clinically euthyroid (16). Similar discrepancies are also noted during treatment with estrogen (17) or testosterone (18) in normal subjects. In order to explain these observations by the classical feed-back theory, the assumption has been made that the level of circulating free thyroxine, rather than the level of protein-bound thyroxine, controls the "pituitary-thyroid axis" (4).

In the present study, trypan blue administration clearly suppressed thyroid weight, thyroidal radioiodine uptake, thyroid hormone secretion, and serum PBI. Several possibilities can be considered in evaluating the effect of trypan blue on the pituitary-thyroid system: (1) a direct inhibitory action of trypan blue on the thyroid gland, (2) a direct inactivation of circulating thyrotropin, (3) a specific inhibitory effect on the secretion of thyrotropin by the adenohypophysis, (4) a non-specific toxic effect on either the pituitary or the thyroid.

The control and the treated animals showed no marked variations in their responses to graded doses of thyrotropin. This strongly suggests that trypan blue does not directly inhibit the thyroid cells or directly inactivate TSH. It is thus most likely that the primary effect of the dye is the inhibition of the secretion of thyrotropin by the pituitary. The slightly smaller pituitary observed in the treated animals could be related to such an effect. The fact that adrenocorticotropin and gonadotropin secretion were not suppressed in these studies suggests a specific effect of trypan blue on thyrotropin secretion.

The decrease in both total and protein-bound serum iodine may be explained by the decreased availability of thyroxine-binding sites on the serum proteins following trypan blue treatment as indicated by Crispell *et al.* (12). More free thyroxine would thus be available and would rapidly penetrate the tissues, including the pituitary, perhaps explaining in part the apparent decrease in TSH secretion.

Several drugs have been shown to produce thyroid hypofunction without a concomitant increase in thyrotropin, as appears to be the case for trypan blue in these studies. Dinitrophenol apparently alters the normal pituitary-thyroid interplay (19, 20), while lithosperm causes both thyroid and testicular atrophy (21). The effect of dinitrophenol is claimed to be due to the liberation of free thyroxine from specific binding sites on serum protein (22). Lithosperm may act as a non-specific toxic agent, or may inactivate tropic hormones in the blood (23). It is possible that the mechanism of action of these three drugs is entirely different, although the end results, so far as thyroid function goes, appear to be the same. The mechanism of action of trypan blue is explored further in the next paper.

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MECHANISM OF ACTION OF TRYPAN BLUE IN SUPPRESSING THYROID FUNCTION[1]

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ABSTRACT

Several of the possible pathways by which trypan blue may affect thyroid function were investigated. The abnormal serum globulin produced by trypan blue injection did not seem to inhibit thyroid activity directly since its appearance did not always coincide precisely temporally with decreased thyroid activity. Direct intrapituitary injection of 0.4 mg. of this protein prepared by continuous flow paper electrophoresis had no effect on thyroidal secretion.

Direct injection of systemically ineffective amounts of trypan blue into the pituitary inhibited thyroid function. The intrahypophyseal injection of small amounts of Evans' blue also suppressed thyroid function.

The binding pattern of labeled thyroxine to the serum proteins of trypan blue-treated animals was quite different from that of normal controls. The amount of thyroxine bound to albumin decreased to 40% of the normal while the thyroxine bound to the protein between α-1 and α-2 globulin, where the abnormal serum protein is located, increased correspondingly. The shift in thyroxine binding seemed to be primarily between the albumin and inter-alpha globulin areas since there was no significant change in the amount of thyroxine bound to the other serum components.

The hypophysis appeared to play an essential role in the appearance of the abnormal serum globulin following trypan blue administration. This globulin would appear when only 1/100th of the minimal systematically effective dose of trypan blue was injected directly in the pituitary. Conversely, the protein did not appear when systemic doses of trypan blue which were effective in the control animals were given to hypophysectomized rats.

IN THE preceding paper it was shown that the administration of trypan blue depressed thyroid function but that the adrenals and testes were not affected (1). It appeared that trypan blue specifically inhibited the secretion of thyrotropin by the adenohypophysis but its exact mechanism of action was not delineated. Trypan blue treatment also produced an abnormal serum component which migrated between α-1 and α-2 globulin at pH 8.6 (2).

In the present study, three experiments were undertaken: (1) the effect of systemically ineffective amounts of trypan blue injected into the pituitary

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tory was studied to determine whether there is a direct inhibition of thyrotropin secretion at the level of the anterior pituitary. (2) The relationship of the abnormal serum protein to a possible increase of free thyroxine following trypan blue treatment was investigated. (3) To clarify the specificity of trypan blue, the effect on thyroid function of the chemically related Evans' blue was studied.

MATERIALS AND METHODS

One hundred thirty-nine adult male Holtzman rats, weighing 200 to 300 gm., were employed. Animals used for the study of thyroid hormone secretion were fed a low iodine diet beginning seven days before the experiment; the other animals were fed Purina laboratory chow. From 0.2 to 20 mg. of trypan blue were dissolved in 4 ml. of physiologic saline and injected intraperitoneally once or twice a day for varying periods of time. Blood was removed from the jugular vein during ether anesthesia and the separated serum was kept frozen until used. Appropriate amounts of radioactive thyroxine (Abbott) were added directly to the serum to obtain concentrations of 5 to 60 μg. of thyroxine per 100 ml. of serum. These samples were gently shaken three times during a period of standing, first at 4° C for 13 hours and then at room temperature for seven hours, to allow binding with serum protein. Serum with or without added labeled thyroxine was developed in the Spinco electrophoretic instrument using a pH 8.6 Barbital buffer. The distribution of radioactivity among the serum components was determined with a strip scanner. Each strip was then stained with Spinco Dye B-1. The concentration of labeled thyroxine among the various components was assessed by planimetry of the strip scanning charts.

Ten ml. of serum from trypan blue-treated rats were subjected to continuous-flow paper electrophoresis to separate the various components. Thirteen tubes were utilized in the continuous electrophoresis and of these #6 gave a relatively pure yield of the abnormal globulin.

Thyroid hormone secretion was measured as previously described (1). Using a microinjection technique employing permanently fixed intracranial hypodermic needles (3), 0.1, 0.2 or 1.0 mg. of trypan blue or Evans' blue, or 0.4 mg. of the abnormal serum component, was injected directly into the pituitary. All test substances were injected in a volume of 0.02 ml. physiologic saline.

Hypophysectomy was performed by the parapharyngeal route. Two days postoperatively, 4 ml. of 0.5% trypan blue in saline were injected intraperitoneally and blood was removed from the jugular vein one to 24 hours after the trypan blue injection. Electrophoresis of the serum thus obtained was performed as described above. Completeness of hypophysectomy was checked at autopsy by examination of the sellar region with a 10× dissecting microscope.

RESULTS

Relationship between the appearance of the abnormal serum component and the suppression of thyroid hormone secretion caused by the administration of graded doses of trypan blue

As seen in Table 1, 10 or 20 mg. of trypan blue resulted in suppressed thyroid function, as measured by I^{131} release rate, and in the appearance of the abnormal serum component. However, when 10 mg. of trypan blue were given, thyroid suppression appeared within one hour but the inter-

TABLE 1. RELATIONSHIP BETWEEN THE ABNORMAL SERUM COMPONENT AND THYROID SUPPRESSION PRODUCED BY TRYPAN BLUE

Dose of trypan blue	Suppression of thyroid function	Appearance of the abnormal component after trypan blue injection					
		1 hour*	6 hours*	12 hours*	24 hours*	3 days†	6 days‡
20 mg.	+	+	+	+	+	+	+
10 mg.	+	±				+	+
4 mg.	−	−				−	−
2 mg.	−	−				−	−
0.2 mg.	−	−				−	−

* = after single injection.
† = once daily for 3 days.
‡ = twice daily for 6 days.

alpha globulin did not appear until later. Doses smaller than 10 mg. were ineffective in producing either thyroid suppression or the abnormal serum component, even after repeated injections twice daily for 6 days. A single injection of more than 10 mg. of trypan blue caused a suppression of thyroid hormone secretion which lasted from 15 to 24 hours, although the abnormal serum component persisted for about 5 days.

The effect of intrapituitary injection of the abnormal serum component on thyroid hormone secretion

Although the presence of the abnormal protein in the serum was not precisely correlated with thyroidal suppression, it seemed possible that it might bear some functional relationship to the changes produced in thyroid activity. For this reason, 0.4 mg. of the relatively pure component obtained by continuous flow electrophoresis was injected directly into the pituitary in 4 animals. As seen in Figure 1, there was no change in thyroid secretion after this treatment.

The effect of intrapituitary injection of systemically ineffective amounts of trypan blue on thyroid hormone secretion and on the appearance of the abnormal serum component

As mentioned above, 10 mg. was the minimal dose which, when injected systemically, would result in thyroid suppression and the appearance of the abnormal serum globulin. Accordingly, 0.1, 0.2 or 1.0 mg. of trypan blue were injected directly into the pituitary in 11 animals. As shown in Figure 2, 1.0 mg. produced an inhibition of thyroid secretion which appeared within one hour and lasted for about 24 hours, an effect very similar to that observed after systemic injection of 10 to 20 mg. Injections of 0.1 and 0.2 mg. trypan blue failed to produce thyroid suppression. Quite interestingly, it was found that the serum from all animals receiving intrapituitary injections of trypan blue contained the abnormal serum protein (Fig. 3). No blue color was detectable in any of the sera.

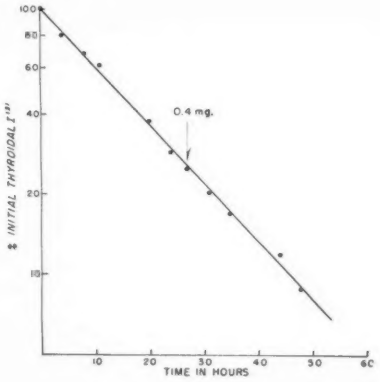

FIG. 1. Effect of intrapituitary injection of abnormal serum component on thyroid hormone secretion.

The effect of hypophysectomy on the appearance of the abnormal serum component

Since the abnormal serum protein would appear when 1/100th of the minimal systemically effective dose of trypan blue was injected directly into the pituitary, it was considered that the hypophysis might have some role in its appearance. To test this hypothesis, 20 mg. of trypan blue were injected intraperitoneally into hypophysectomized animals and one to

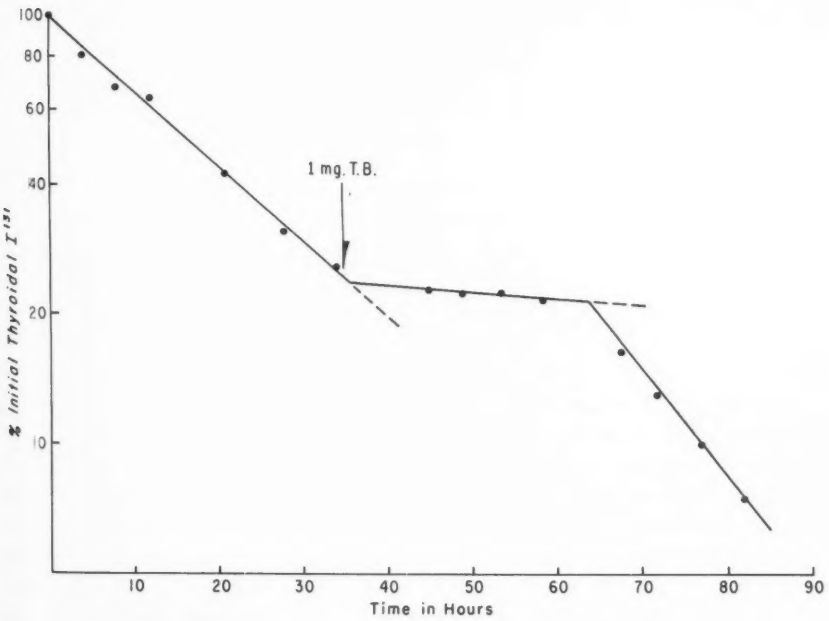

FIG 2. Effect of intrapituitary injection of trypan blue on thyroid hormone secretion.

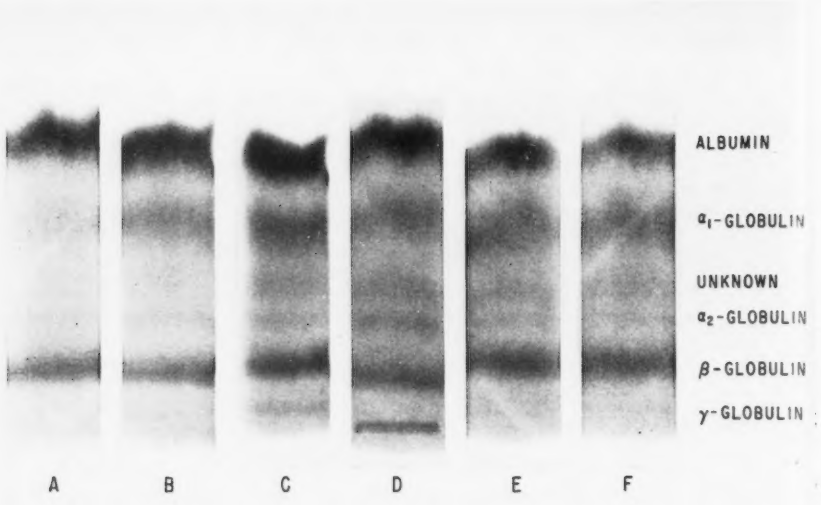

FIG. 3. Paper electrophoresis of rat serum. A = saline injected control, B = 4 mg. systemic injection of T.B. twice daily for 6 days, C = 10 mg. systemic injection of T.B. once daily for 3 days, D = 1 hour after single injection of 20 mg. T.B. systemically, E = 0.1 mg. intrapituitary injection of T.B., F = 0.1 mg. intrapituitary injection of E.B. C, D, S and F have an abnormal component between α-1 and α-2 globulin. Trypan blue stayed at the origin.

24 hours later the serum was analyzed. The abnormal protein did not appear in completely hypophysectomized animals but it did appear in those in which the pituitary was not completely removed (Fig. 4).

Thyroxine-binding capacity of normal and trypan blue-treated rats

In normal rat serum, radioactive thyroxine added *in vitro* was bound to all serum proteins, even when small amounts were added, but maximal concentration was seen consistently in the albumin portion. As indicated in Table 2 and Figure 5, when the concentration of added thyroxine was 5 or 20 μg. per 100 ml. of serum, the amount of thyroxine binding with albumin was markedly decreased after trypan blue treatment while thyroxine binding between α-1 and α-2 globulin increased. The amount of thyroxine bound to the other serum fractions was the same in both the trypan blue-treated and normal rats.

A significant change in localization of labeled thyroxine was produced when the concentration was increased to 60 μg. per 100 ml. serum. The greatest concentration was seen between α-1 and α-2 globulin in the trypan blue animals. In the control animals, however, two prominent peaks were seen, one between α-1 and α-2 globulin and one in albumin or between albumin and α-1 globulin (Fig. 6).

TABLE 2. DISTRIBUTION OF RADIOACTIVE THYROXINE AMONG SERUM COMPONENTS

	5 μg./100 ml. level of added thyroxin			20 μg./100 ml. of added thyroxin		
	Control	Trypan blue treated		Control	Trypan blue treated	
Albumin	36.8 (±2.1)*	14.5 (±1.6)†		37.2 (±3.4)	19.7 (±2.5)	
Albumin-α-1 Globulin	13.8 (±1.9)	19.9 (±1.5)		15.5 (±0.9)	22.3 (±0.5)	
α-Globulin	22.6 (±1.8)	23.6 (±1.4)		22.3 (±0.5)	21.0 (±1.2)	
α-Globulin-Unknown Compound		10.6 (±0.9)			10.0 (±0.7)	
Unknown Compound	18.9 (±2.1)	12.2 (±0.8)	31.9	20.7 (±0.8)	13.8 (±1.1)	33.7
Unknown Comp.-β-Globulin		9.1 (±0.5)			9.9 (±0.5)	
β-Globulin	4.3 (±0.9)	5.5 (±0.2)		3.4 (±0.6)	5.9 (±0.7)	
β-Globulin-Origin	2.6 (±0.7)	3.4 (±0.7)		2.0 (±0.5)	3.8 (±0.6)	
Origin	1.0 (±0.5)	1.4 (±0.9)		0.1 (±0.1)	0.9 (±0.4)	

*=Mean and standard error calculated from the values of 4 animals.
†=Mean and standard error calculated from the values of 6 animals.

The effect of intrapituitary injection of Evans' blue on thyroid hormone secretion and on the appearance of the abnormal serum component

To ascertain whether the effects of trypan blue listed above were due to its specific action or were a common property of similar diazo dyes, graded doses of Evans' blue were injected into the pituitary in 6 rats and the effect on thyroid hormone secretion observed. One mg. of Evans' blue produced thyroid suppression (Fig. 7) but amounts less than that were

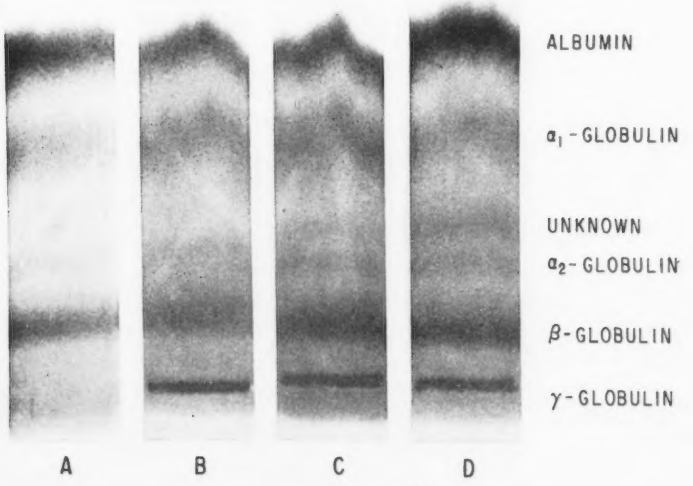

FIG. 4. Effect of hypophysectomy on the electrophoretic pattern of the serum in trypan blue-treated rats. The blood was taken 24 hours after T.B. injection. A=saline injected control, B=trypan blue-treated, completely hypophysectomized animal, C=trypan blue-treated, incompletely hypophysectomized animal, D=trypan blue-treated normal animal. The abnormal component appeared in C and D.

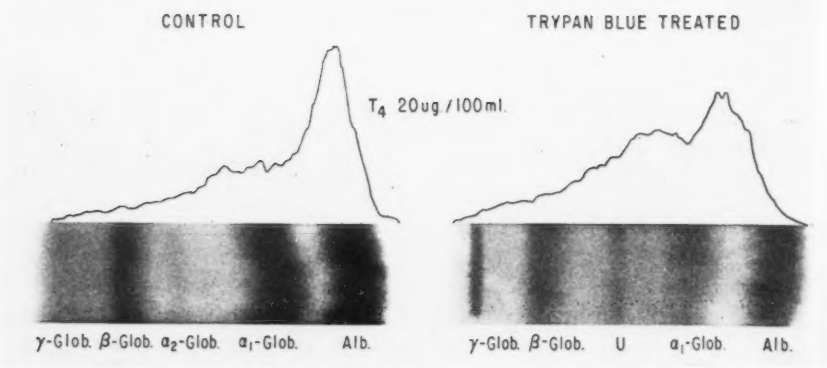

FIG. 5. Paper electrophoresis of a mixture of animal sera and labeled L-thyroxine, concentration: 20 μg. T_4/100 ml. serum. In the normal controls most of the thyroxine bound with albumin. In the trypan blue-treated animals thyroxine binding to albumin was reduced by $\frac{2}{3}$ and there was a marked increase in binding between α-1 and α-2 globulin. Alb = albumin, α-1 Glob. = α-1 globulin, U = abnormal component, α-2 Glob. = α-2 globulin, β-Glob. = β-globulin, γ-Glob. = γ-globulin. Trypan blue stayed at the origin.

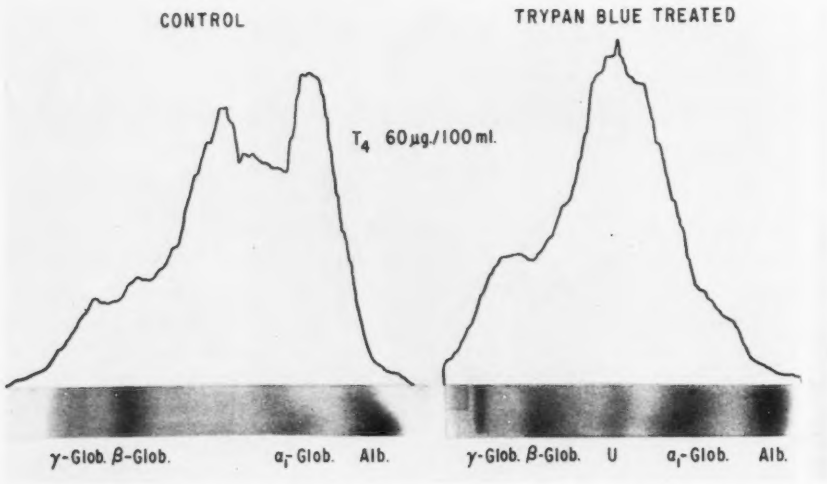

FIG. 6. Paper electrophoresis of a mixture of animal sera and labeled L-thyroxine, concentration: 60 μg. T_4/100 ml. serum. In the normal animals, thyroxine was bound not only to albumin and α-1 globulin, but also to α-globulin. In the trypan blue-treated animal, almost all thyroxine was bound to α-globulin. Abbreviations as in Figure 5.

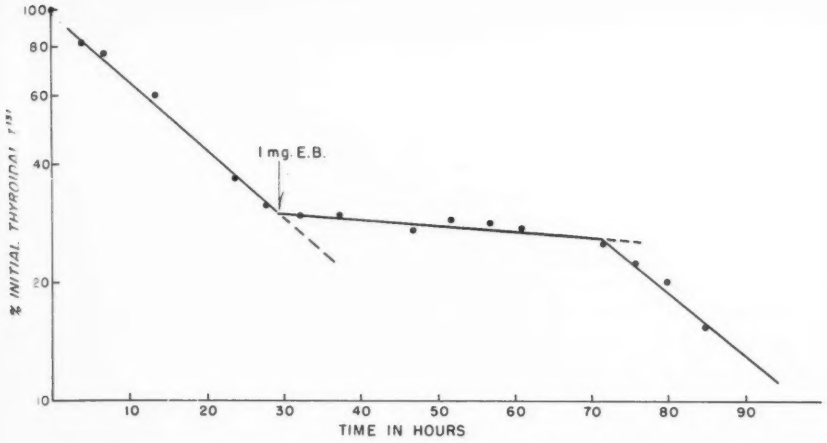

FIG. 7. Effect of intrapituitary injection of Evans' blue on thyroid hormone secretion

ineffective. The abnormal serum component appeared with all doses of Evans' blue used (Fig. 3), although no blue dye was detectable in the sera.

DISCUSSION

In the preceding paper it was shown that the trypan blue-suppressed thyroid would respond normally to exogenous TSH (1). It was deduced that trypan blue might suppress thyrotropin secretion by the adenohypophysis and that secondary hypofunction of the thyroid would thus result. In the present study, this hypothesis was strengthened by finding that $\frac{1}{16}$ of the minimal systemically effective dose of trypan blue would suppress thyroid function when injected directly into the pituitary. The mechanism of this action was then investigated.

Robbins and Rall (4) have indicated that the thyroxine binding protein of human serum migrates between α-1 and α-2 globulin when electrophoresis is carried out on paper strips at pH 8.6 in Barbital buffer. Very recently Rabinovitch *et al.* (5, 6) have shown that bovine fraction VI has a component, possibly a glycoprotein, which is capable of binding thyroxine to the exclusion of other components, and which markedly inhibits conversion of the inorganic iodide ion in the rat to organic protein-bound iodine. In the present study, the abnormal serum component which seemed to appear with the onset of thyroid suppression following trypan blue administration had an electrophoretic mobility between α-1 and α-2 globulin at pH 8.6 in Barbital buffer and actually did bind thyroxine. Therefore it was first considered that the abnormal serum globulin might be an important factor in producing thyroid suppression. However, it was found that the appearance of this component did not always coincide precisely with the appearance of thyroid suppression. Furthermore, direct

intrahypophyseal injection of rather large amounts of this protein had no effect on thyroid hormone secretion. It thus appears that this abnormal protein does not directly produce thyroidal suppression.

It is of interest that systemically ineffective amounts of trypan blue will cause the appearance of this abnormal serum globulin when injected directly into the pituitary and that hypophysectomy will prevent its appearance, even when large amounts are injected systemically. It is possible that some pituitary hormone directly stimulates the formation of the serum component or that the hypophysis plays a "permissive" role in its formation following trypan blue treatment. Since systemically ineffective amounts injected directly into the pituitary will result in the appearance of this protein, at present it seems more likely that there may be a direct hypophyseal action in its formation.

It was also considered that an increase of free thyroxine produced by competition by trypan blue for binding sites on serum protein might explain the suppression of thyroid function. The binding of thyroxine to serum protein was found to be quite different in rat serum than in human serum, in which thyroxine-binding protein or pre-albumin is the primary carrier of thyroxine and albumin is the secondary carrier. In normal rat serum, 40% of added thyroxine is bound to albumin when the thyroxine concentration is 5 or 20 μg. per 100 ml. of serum. This agrees with the reports by Robbins and Rall (4) and by Kalant *et al.* (7). When the quantity of added thyroxine was increased, thyroxine was found to bind with both albumin and with the protein between α-1 and α-2 globulin. These results are exactly the opposite of what has been found in the human, and indicate that in the rat albumin is the primary thyroxine carrier and the protein between the α-1 and α-2 globulin is the secondary carrier. In the sera of trypan blue-treated rats, the amount of thyroxine bound to albumin was 40% of the normal value, and the amount bound to the protein between α-1 and α-2 globulin, where the abnormal serum protein was present, was correspondingly increased. The shift seemed to be primarily between albumin and the inter-alpha globulin area, no significant change being found in the amounts of thyroxine bound to other serum components. The decreased amount of thyroxine bound to albumin in the trypan blue-treated rats may be due to the decreased quantity of albumin in these animals, but a change in the binding capacity of albumin may also be implicated.

It is thus apparent that thyroidal hypofunction coincided with marked changes of the thyroxine-binding pattern of the serum protein. However, this fact would not necessarily indicate an increase of free thyroxine since there is as yet no actual proof that the thyroxine-binding capacity of the inter-alpha globulin is weaker than that of albumin and that thus more free thyroxine may be available to the cells following trypan blue treat-

nent. The disappearance rate of labeled thyroxine from the blood, which would give more information, is not known at the present time.

Crispell *et al.* found that trypan blue would interfere with the thyroxine-binding capacity of serum protein *in vitro*, but that Evans' blue was ineffective (8). However, in the present experiments it was found that both Evans' blue and trypan blue suppressed thyroid secretion when injected into the pituitary. It is thus uncertain whether trypan blue produces thyroid suppression due to a common toxic action of diazo dyes on the pituitary or whether it has a specific effect which increases the amount of free available thyroxine. Any "toxic" effect of trypan blue on the pituitary seems to affect only thyrotropin secretion since ACTH and gonadotropin secretion apparently are not impaired.

Acknowledgments

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VARIATIONS IN HEPATIC INACTIVATION OF CORTICOSTERONE WITH CHANGES IN FOOD INTAKE: AN EXPLANATION OF IMPAIRED CORTICOSTEROID METABOLISM FOLLOWING NOXIOUS STIMULI[1,2]

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ABSTRACT

In studies involving nine different noxious stimuli, all stimuli which diminished food intakes of male or female rats also diminished the capacity of the liver to inactivate adrenal cortical hormones by ring A reduction. All noxious stimuli which failed to affect food consumption also failed to affect corticosterone metabolism. In every case, the loss of hepatic capacity for inactivation of corticosterone following exposure to noxious stimuli could be duplicated exactly in control animals pair-fed to those exposed to the noxious stimuli. Hepatic Δ^4-steroid hydrogenase activity was found to be a nearly linear function of food intake in the 18 hours immediately preceding the assay.

Variations in water intake, and in ACTH or corticosterone secretion rate or concentration were not involved in the loss of hepatic Δ^4-steroid hydrogenase activity following exposure to noxious stimuli.

Three *in vivo* experiments demonstrated that variations in the rate of ring A reduction of adrenal cortical hormones by liver *in vitro* accurately predict variations in the overall rate of corticosteroid metabolism in intact animals.

IMPAIRMENT of hepatic function or blood flow often follows anesthesia and surgical trauma in man (2, 3). Several types of indirect evidence have suggested that one aspect of impaired hepatic function under these conditions is diminution in the rate of inactivation of adrenal cortical hormones. The evidence includes observations that: plasma concentration of adrenal cortical hormones after surgical trauma may be greater than can be produced by exogenous adrenocorticotropin (ACTH) in the absence of trauma (4); the increment, or the rate of rise, of plasma concentration of corticosteroids following injection of adrenal cortical hormones or ACTH may be augmented in the presence of trauma (5, 6); the rate of disappearance of injected corticosteroids from plasma is slowed in surgical

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patients, in terminally ill patients, and in anesthetized dogs subjected to hemorrhage in combination with intravenous pyrogens or exposure to cold or heat (6–8); and that the rise in plasma corticosteroid concentration after surgery is highest in those patients showing the greatest retention of bromsulfophthalein post-operatively (9).

In contrast, evidence that the metabolism of adrenal cortical hormones in rats is *not* altered by anesthesia, forced exercise, epinephrine or histamine has been reported by Ulrich and Long (10). Neither the half-life of corticosterone-4-C^{14} in plasma after a single injection, nor the levels reached during constant infusion was affected by these stimuli in normal or adrenalectomized rats.

The nature and cause of impaired hepatic inactivation of corticosteroids apparently associated at least occasionally with anesthesia, disease or trauma have not been identified. In man, trauma causes an impairment in the dehydrogenation of the 11-hydroxyl group of cortisol (11), but it is uncertain whether impaired conversion of cortisol to cortisone can be considered as an impaired inactivation of cortisol.

The present experiments were designed to examine the effects of noxious stimuli on corticosterone metabolism in the rat, in order to discover which noxious stimuli impair hepatic inactivation of adrenal cortical hormones, and whether these have any other features in common. From earlier work we have concluded that ring A reduction of corticosteroids by liver represents the physiological process of inactivation of these hormones in rats better than does any other single reaction (12–14), and so the Δ^4-steroid hydrogenase system was chosen for study in the present experiments. We have here defended this choice by showing that changes in the capacity of the liver to inactivate corticosterone by ring A reduction *in vitro* are associated with the appropriate changes in the biological half-life of corticosterone *in vivo*.

METHODS

Male or female rats of the Charles River Laboratory Caesarean-derived strain were used in all experiments. They were kept in thermoregulated rooms (25° C) and maintained on Purina chow and tap water *ad libitum* until experiments were begun. The assays of liver slices and homogenates for Δ^4-steroid hydrogenase activity were performed as previously described (14, 15). Corticosterone was used as substrate in all assays. Plasma corticosterone concentrations were measured in 0.5 ml. samples by the modification of the Sweat sulfuric acid fluorescence method which has been described by Guillemin *et al.* (16).

The *in vivo* half-life of corticosterone-4-C^{14} in plasma was determined by the method of Ulrich and Long (10) with slight modification. At time zero each animal received 50 μg of corticosterone intravenously, containing 0.2 μc., or 150,000 cpm. as measured in our system, of the labelled steroid. Six 0.3 ml. venous blood samples were withdrawn at approximately ten-minute intervals from another site. After centrifugation of the blood, 0.1 ml. plasma samples were plated on lens paper to reduce self-absorption, and counted for 2,000 counts in a helium-butane quenched gas flow counter operating in the Geiger

range. The least squares line relating the log cpm./0.1 ml. plasma to time elapsed was used to calculate the half-life of the corticosterone.

The groups of animals studied are described below.

Controls: Undisturbed animals allowed food and water *ad lib.* until the moment of assay.

Croton oil granuloma pouch: 0.5 ml. of 2% croton oil (diluent, sesame oil) injected subcutaneously into a 25 ml. air pocket between the scapulae, after the method of Selye (17). Assayed 48 to 96 hours later.

Surgical trauma: Laparotomy or splenectomy under ether anesthesia at approximately 4:00 P.M. Assayed at 10:00 the following morning.

Overnight fast or partial fast: Graded amounts of food, from none to an excess above the normal ration, supplied between 4:00 P.M. and 10:00 the following morning. Assayed at 10:00 A.M.

Overnight at 0° C: Animals placed singly in cold at 4:00 P.M., allowed food and water *ad lib.* Assayed at 10:00 the following morning.

Hypoxia (equivalent to altitude of 15,000 feet above sea level): Animals placed for three days in a sealed glass chamber with continuous flow of 11% oxygen and 89% nitrogen at 3.75 liters/min. The steady-state oxygen tension was 90 mm. Hg; the CO_2 fraction in inspired air was 0.3%—determined by a Pauling oxygen meter and a Mead CO_2 analyzer. The ambient barometric pressure ranged from 757 to 774 mm. Hg, the dry bulb temperature was 77° F, the wet bulb temperature at the specified gas flow rate was 65° F and the relative humidity was 52%. Food and water were offered *ad lib.* The rats were removed from the chamber for 30 minutes out of each 24 hours for cage cleaning. The steady-state conditions described were re-established within 20 minutes after returning the rats to the chamber.

Anesthesia: Rat anesthetized with ether and then allowed to recover, once every 15 minutes, during the two-hour period immediately prior to assay.

Exercise: Forced swimming in water at 25° C for three hours, followed immediately by assay.

Proximity to cat: Rats placed singly at 4:00 P.M. in wire-mesh metabolism cage which was secured inside a larger cat cage, containing a fasted tomcat. The rat was offered food and water *ad lib.* until assay at 10:00 the following morning.

Force-fed groups: Animals force-fed a complete, concentrated human baby food supplying 20 kcal./30 ml., and containing 5.4% (w/v) protein and 5.6% (w/v) fat. The diet was forced by stomach tube in 15 ml. amounts every few hours during the night before assay, to a total dose equal to 25% of the initial body weight (v/w). The same fluid volume of tap water was similarly forced into a control group. The liquids were warmed to 38° C before being forced. No other source of food or water was offered.

Unit of enzymatic activity

Experiments involving changes in food intake inevitably involve changes in body weight and liver weight, and so render the expression of the activity of hepatic enzymes somewhat ambiguous. In all the figures we have expressed the activity of the Δ^4-steroid hydrogenase in the liver as uM of corticosterone (B) reduced at ring A per minute, per 100 grams of body weight at the time of assay. This activity unit is affected by changes in liver weight, in body weight, and in the enzymatic activity per unit weight of liver. For calculation and discussion of this unit see reference (14). In order to clarify the meaning of changes in this derived unit of activity, the raw data showing the initial and final body weights, the liver weight at assay, and the enzymatic activity per 200 mg. of liver tissue are given for all female groups in Table 1, and for all male groups in Table 2.

Data presented as means are accompanied by the standard errors of the means.

RESULTS

In the early experiments we found that noxious stimuli of short duration, such as repeated anesthetization with ether for two hours, or forced swimming for three hours, had no effect on the *in vitro* reduction of ring A of corticosterone by liver slices, but that more prolonged stimuli, such as proximity to cat overnight, or overnight fast, caused severe losses of hepatic Δ^4-steroid hydrogenase activity. Since overnight fast itself impaired the inactivation of corticosterone, the effects of all other noxious stimuli on food intake were determined in order to discover whether those which impaired corticosterone metabolism acted by provoking a partial or complete fast, or by some other means.

We first determined the diurnal cyclic variation of food intakes in rats kept in our laboratory. The results are shown in Figure 1. In most of the subsequent experiments the control or measurement of food intake was begun at 4:00 P.M., at a time when the rats had been on a spontaneous

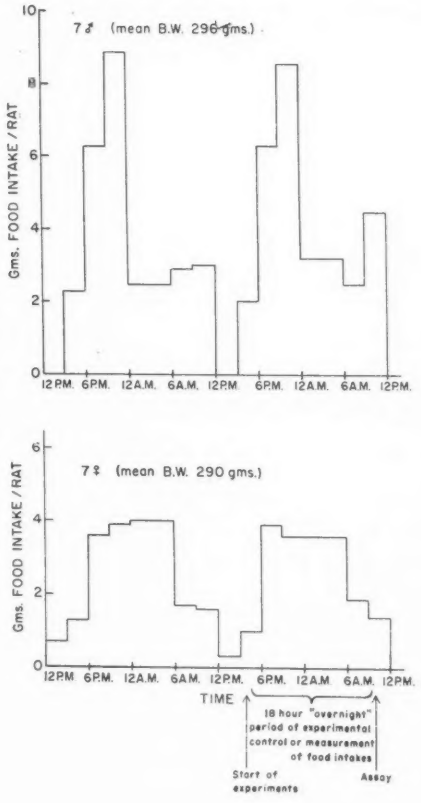

FIG. 1. Diurnal cycle of food intakes in control male and female rats during two consecutive days. Subsequent experiments were designed so that food intakes of all groups were measured between 4:00 P.M. and 10:00 A.M. the following morning, at which time the assays were performed.

reduction of food intake for approximately 12 hours. Thus, what we have called an overnight fast consists of approximately 12 hours of spontaneous partial fast, followed by 18 hours of enforced total fast, as indicated in Figure 1.

Control groups

Livers from female rats reduce ring A of adrenal cortical hormones at a much faster rate than do those from males (control groups, Figs. 2 and 3) (13, 18–20). This large sex difference in corticosteroid metabolism is associated with, and possibly causes, a large sex difference in the *in vivo* half-life of labelled corticosterone in plasma of intact rats. The half-life of corticosterone-4-C^{14} in female rats is approximately 11.3 minutes (Fig. 6) while that in male rats is approximately 22.2 minutes (not shown).

The hepatic capacity for the inactivation of corticosterone in all groups was unaccountably lower in later experiments than in the earlier ones (for example, compare the male control groups shown in Figs. 3 and 7, and in Table 2A and 2B). The per cent change in activity in any given experimental group was the same after the shift had occurred as it was before,

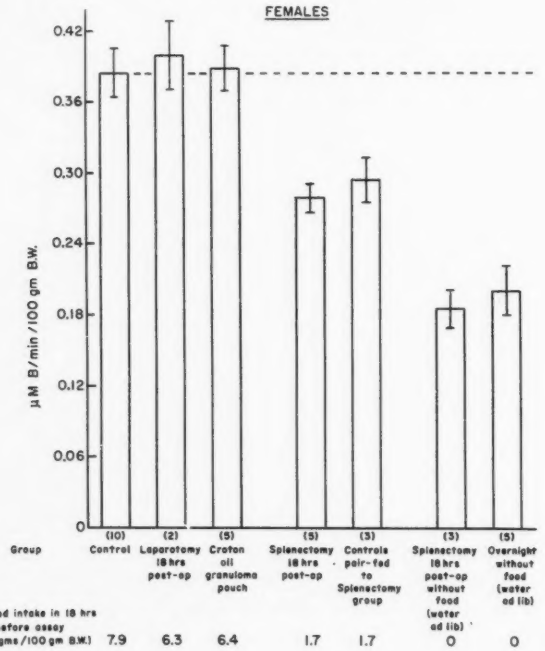

FIG. 2. Effects of noxious stimuli on the inactivation of corticosterone by liver slices from female rats. Those stimuli which involved substantial reductions in food intake also caused impairment in the reduction of ring A of corticosterone.

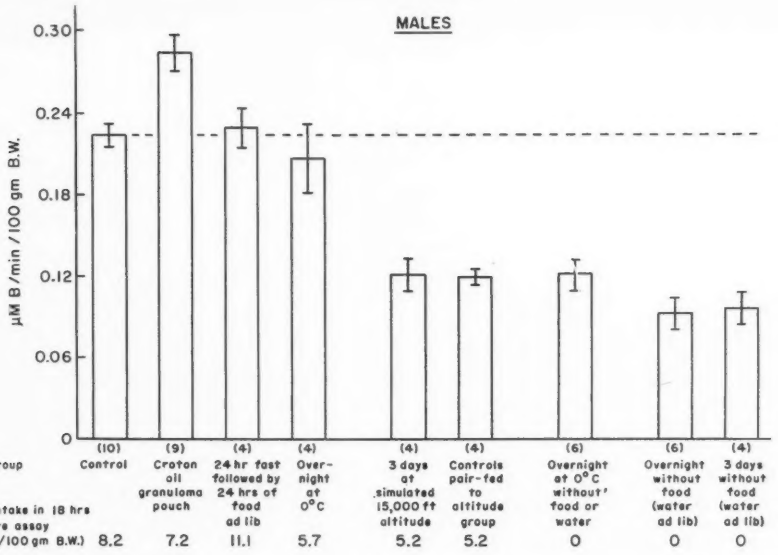

FIG. 3. Effects of noxious stimuli on the inactivation of corticosterone by liver slices from male rats. Those stimuli which are associated with a substantial diminution of food intake also cause impairment in the reduction of ring A of corticosterone. The average food intake of the altitude group and its pair-fed control over the whole three-day experimental period was 2.0 gm./100 gm. B.W.

and so the relative results are not affected by this unexplained change in absolute levels of activity. Experimental groups are presented with their simultaneously assayed controls in every case.

Effects of noxious stimuli in females

The results are shown in Figure 2 and Table 1. Overnight fast caused a 52% loss of hepatic Δ^4-steroid hydrogenase activity. Splenectomy reduced the food intake of the rats, and resulted in a diminution of enzymatic ac-

TABLE 1. RAW DATA SHOWING EFFECTS OF VARIOUS NOXIOUS STIMULI ON BODY WEIGHT, LIVER WEIGHT, AND ACTIVITY OF Δ^4-STEROID HYDROGENASE PER UNIT WEIGHT OF LIVER (THE ACTIVITY CONCENTRATION) IN FEMALE RATS

Group (females)	No.	Initial B.W. (gm.)	B.W. at assay (gm.)	Liver weight at assay (gm.)	Liver slice activity uM cpd, B/200 mg. wet wt. /15 min.
Control	10		239 ± 12	9.9 ± 1.6	0.274 ± .009
Laparotomy	2		247	9.3	0.319
Croton Oil Granuloma Pouch	5		298 ± 6	12.1 ± 1.4	0.285 ± .010
Splenectomy	5	243 ± 5	235 ± 7	8.1 ± 0.34	0.250 ± .019
Controls pair-fed to splenectomized group	3	251 ± 6	237 ± 13	8.4 ± 0.3	0.252 ± .012
Splenectomy plus overnight fast	3	239 ± 6	228 ± 6	6.6 ± 1	0.198 ± .016
Overnight fast	5	236 ± 5	221 ± 5	6.9 ± 0.3	0.193 ± .014

TABLE 2. RAW DATA SHOWING EFFECTS OF VARIOUS NOXIOUS STIMULI ON BODY WEIGHT, LIVER WEIGHT, AND ACTIVITY OF Δ^4-STEROID HYDROGENASE PER UNIT WEIGHT OF LIVER (THE ACTIVITY CONCENTRATION) IN MALE RATS

The A and B components of the table indicate the controls and experimental groups to be compared. An unexplained shift in control values occurred during a six-month period between the experiments under A and those under B.

Group (males)	No.	Initial B.W. (gm.)	B.W. at assay (gm.)	Liver weight at assay (gm.)	Liver slice activity uM cpd. B/200 mg. wet wt. /15 min.
A. Early experimental groups					
Control	10		319 ± 16	14.3 ± 0.8	0.154 ± .009
Croton Oil Granuloma Pouch	9		345 ± 8	14.6 ± 0.5	0.207 ± .006
24-hr. fast followed by 24 hrs. of food *ad lib.*	4	289 ± 8	288 ± 9	13.7 ± 0.8	0.145 ± .007
Overnight at 0° C with food	4	385 ± 9	378 ± 10	15.3 ± 1.1	0.159 ± .016
Simulated 15,000 ft. altitude	4	327 ± 8	340 ± 8	11.2 ± 1.1	0.115 ± .010
Controls pairfed to altitude group	4	303 ± 10	315 ± 8	11.3 ± 0.3	0.098 ± .006
Overnight at 0° C without food or water	6	377 ± 9	307 ± 24	10.2 ± 0.7	0.113 ± .015
Overnight without food; water *ad lib.*	6	318 ± 7	297 ± 12	10.0 ± 0.5	0.084 ± .013
Three days without food; water *ad lib.*	4	265 ± 7	216 ± 3	7.4 ± 0.2	0.082 ± .007
B. Later experimental groups					
Control	12		223 ± 5	9.8 ± 0.4	0.109 ± .003
Overnight without food; water *ad lib.*	5		285 ± 4	9.0 ± 0.4	0.065 ± .006
Overnight without food; water forced by stomach tube	8	272 ± 13	239 ± 13	8.1 ± 0.4	0.070 ± .009
Overnight without usual chow; liquid diet forced by stomach tube	3	318 ± 5	310 ± 9	10.7 ± 0.5	0.160 ± .004
Rapid splenectomy	5	292 ± 24	267 ± 5	9.6 ± 0.3	0.138 ± .009
Slow splenectomy	5	316 ± 28	297 ± 5	11.0 ± 0.2	0.057 ± .013
Slow splenectomy plus force-feeding	7	294 ± 14	301 ± 16	12.8 ± 0.7	0.119 ± .012
Chronic hypophysectomy	6		182 ± 8	5.9 ± 0.2	0.079 ± .003
Chronic hypophysectomy plus overnight fast	6		170 ± 7	4.7 ± 0.2	0.052 ± .003

tivity which could be duplicated exactly by pair-feeding control animals to the splenectomized group. The combination of splenectomy plus enforced overnight fast had the same effect as did the complete fast alone.

Laparotomy and croton oil granuloma pouches had little effect on food intakes, and failed to impair corticosterone metabolism.

Effects of noxious stimuli in males

The results are shown in Figure 3. Animals without food for 18 hours showed a 60% loss of hepatic activity, which was not changed further by prolonging the fast for two more days.

Rats which were hypoxic for three days ate very irregularly during the experimental period. As shown in Figure 3, in the period immediately before assay their food intake was 37% less than that of control rats. In the first two days of the experiment their food intake was reduced even more severely. These animals had approximately a 50% loss in hepatic activity for ring A reduction after the third day at oxygen tensions equivalent to an

ltitude of 15,000 feet above sea level. Control rats pair-fed to the irregular ood intake pattern of the hypoxic rats showed exactly the same degree of mpairment of steroid metabolism as did the hypoxic rats.

Male rats kept overnight at 0–5° C with food and water had a 30% reuction of food intake, associated with occasional freezing of the water upply, but had normal hepatic activity for ring A reduction of corticoserone. The group kept at the same temperature without food and water ad a 50% loss of enzymatic activity—not significantly different from the oss observed after overnight fast alone.

The croton oil granuloma pouch had little effect on food intake of male ats, but nevertheless caused a slight increase ($P < 0.01$) in the rate of inactivation of corticosterone. The cause of this increase is not known, and the increase was not present in similarly treated females (Fig. 2). In another group of males, a 24-hour fast followed by 24 hours of food and water *ad lib.* resulted in an augmented food intake in the period prior to assay, but did not affect the rate of inactivation of corticosterone. This latter group demonstrates that the impaired inactivation of corticosterone following an overnight fast is readily reversed by subsequnt food intake.

Relation between food intake prior to assay, and hepatic capacity for inactivation of corticosterone

In order to examine more precisely the relationship between food intake and steroid metabolism suggested by the preceding experiments, we studied five groups of male rats which were offered graded rations of food ranging from none to an amount greatly in excess of the normal overnight food consumption of these rats. These rations were provided during the 18 hours immediately preceding the assays. Prior to that time the rats had been consuming food and water *ad libitum*. The results are shown in Figure 4. Hepatic Δ^4-steroid hydrogenase activity in slices is a linear function of the food intake during the overnight period before the assay, over a wide range of food intakes.

Figure 5 and Tables 1 and 2 present the data from both sexes which reveal the various components of this close relationship between food intake and hepatic capacity for ring A reduction of corticosterone. An overnight fast causes a very slight decrease in body weight, and a 25% loss of liver weight in both sexes. In the slice assays the enzymatic activity per unit weight of liver (Tables 1 and 2) is decreased 45% in males and 30% in females following overnight fast. Therefore the total loss of activity in slices of 58% in the males, as shown in Figure 5, consists of a large loss of activity per unit weight, and a lesser decrease in total weight of the liver. The total loss of 48% in females consists equally of a loss of activity per unit weight, and a decrease in total weight of the liver.

Since the reaction rate per unit weight of liver in slice assays is determined both by the amount of enzyme present, and by the availability of the

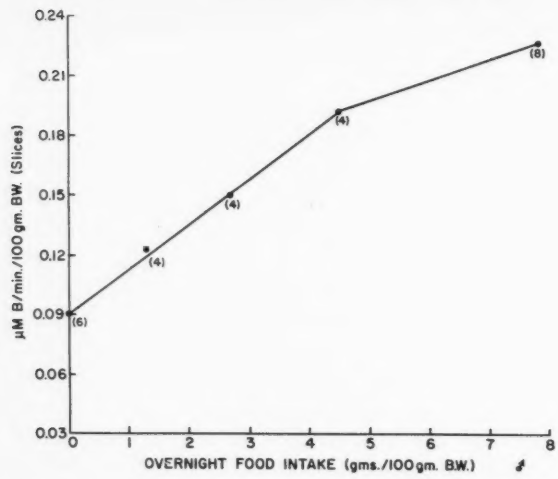

FIG. 4. The relationship between the overnight food intake prior to assay and the Δ^4-steroid hydrogenase activity measured in liver slices from male rats.

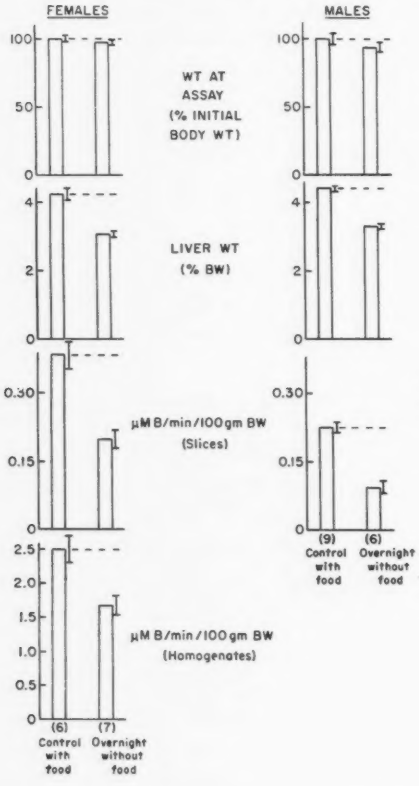

FIG. 5. Analysis of the enzymatic basis for the close relationship between food intake and corticosterone inactivation.

enzyme, reduced triphosphopyridine nucleotide (TPNH), we repeated these experiments in females using an homogenate assay in which the kinetics are zero order with respect to substrate concentration, and in which the enzyme is supplied in excess of requirements (15). This assay therefore estimates changes in amount of enzyme, and is unaffected by changes in availability of TPNH. The results are shown in the bottom of Figure 5. A 25% decrease in the total amount of the enzyme in females fasted overnight was accounted for entirely by a 25% reduction in total liver weight. The amount of enzyme per gram of liver was unchanged.

Relation between hepatic Δ^4*-steroid hydrogenase activity* in vitro, *and the half-life of corticosterone* in vivo

To determine whether the effects of variations in food intake on corticosterone metabolism measured *in vitro* were reflected by appropriate changes in the half-life of corticosterone in intact rats, the half-life of the radioactivity in rat plasma after a single intravenous injection of 0.2 μc. of corticosterone-4-C^{14} was measured in fed and fasted female rats. Under the conditions of these experiments the radioactivity counted in whole plasma represents mainly corticosterone, although in the later samples the combined radioactivity of various metabolites of corticosterone occasionally approaches that of the remaining corticosterone itself (Glenister and Yates—unpublished results).

The biological half-life of corticosterone in the control, fed group was 11.3 ± 0.52 minutes and in the overnight fast group was 14.9 ± 0.73 minutes ($P < 0.01$, Fig. 6). Thus, the 48% loss of Δ^4-steroid hydrogenase activity observed *in vitro* with slices of livers from fasted female rats is associated with a 32% increase in the *in vivo* half-life of corticosterone in fasted, intact female rats.

Dependence of hepatic inactivation of corticosterone on food intake but not on water intake

Rats deprived of food drink little water, and when food is available the amount of water consumed is closely related to the amount of food eaten (21). For this reason it was necessary to determine whether the demonstrated close relationship between food intake and hepatic Δ^4-steroid hydrogenase activity might not actually have represented a relationship between water intake and the enzymatic activity. Four groups of rats were studied. The first group (control) was allowed food and water *ad lib.* until time of assay. The second group (overnight fast) had no food during the 18 hours before assay, but water was available at all times. In the third group food and water were removed at 4:00 P.M., 18 hours before assay, and then during the night a total volume of water equal to 25% of their initial body weights was forced into the rats by stomach tube in five divided doses, three hours apart. The water was warmed to 38° C before it was forced.

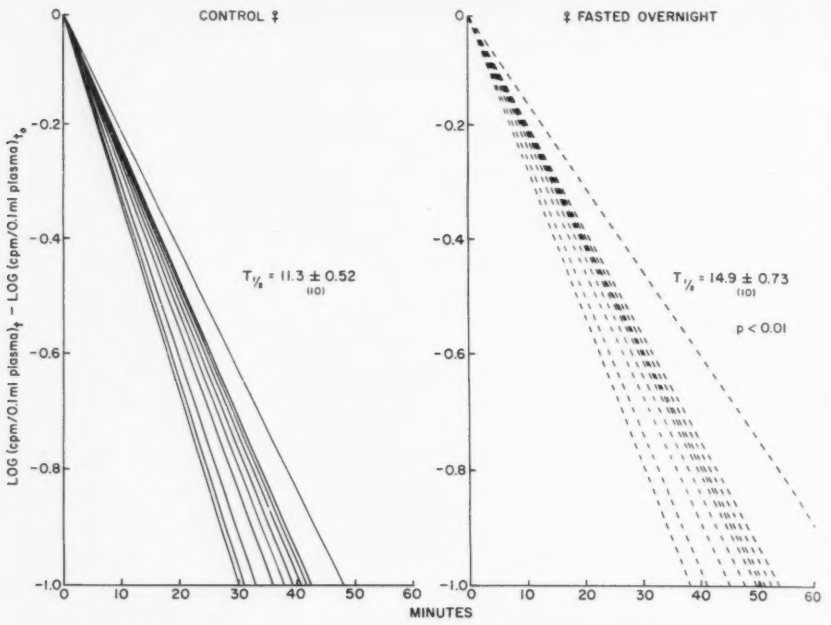

FIG. 6. Effects of overnight fast on the *in vivo* half-life of corticosterone in the plasma of female rats. Each line in the figure represents the least-squares line fitting six experimental points from a single rat.

The animals did not shiver, and the ensuing water diuresis was sufficient to prevent water intoxication. These rats were alert and active at the time of assay, and at autopsy the gastrointestinal tracts were empty. The fourth group received a volume of concentrated, liquid human baby food equal to 25% of their body weights, given in the same manner as was the water to the third group. Formless, semi-solid, yellow feces appeared after the third dose, and at the time of assay the lumen of the whole gastrointestinal tract was filled with curds in every rat. These animals did not look well, and were lethargic.

The results are shown in Figure 7. The overnight fast caused a 56% loss in enzymatic activity, similar to that shown previously (Figs. 3 and 5). The forcing of the large volume of water to animals fasted overnight did not prevent the loss in enzymatic activity associated with the fast, even though the absorption of such large volumes of water presumably provoked an increased splanchnic and hepatic blood flow.

In contrast, the force-feeding of the liquid diet, in a volume equal to that of the water load in the third group, completely prevented the loss in hepatic Δ^4-steroid hydrogenase activity in animals without access to laboratory chow for 18 hours prior to assay.

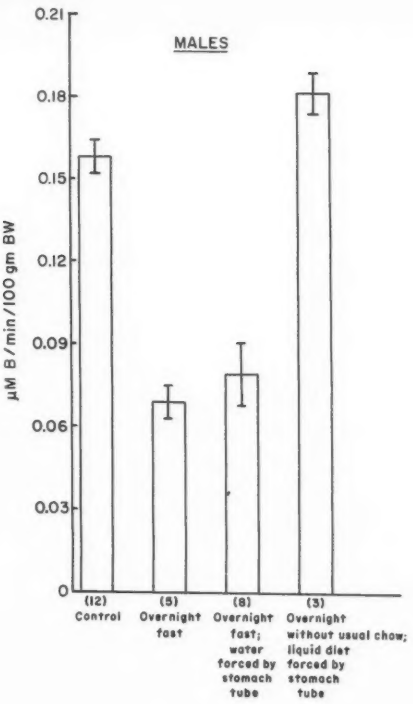

FIG. 7. Dependence of the Δ^4-steroid hydrogenase activity of liver slices upon food intake, or food plus water intake. The activity is completely independent of the water intake alone.

Evidence that the decrease in hepatic inactivation of corticosterone following noxious stimuli is independent of anterior pituitary or adrenal cortical hormones

The discovery by Berliner and Dougherty that ACTH appears to impair the glucosiduronic conjugation of adrenal cortical hormones under certain conditions (22), suggested the possibility that reductions in food intake might provoke release of ACTH, which might then cause the impairment of hepatic inactivation of corticosterone by ring A reduction which was observed in the present experiments.

We performed two experiments to test this possibility (Fig. 8). In the first, severe surgical trauma was produced in ether-anesthetized male rats by splenectomy and handling of the abdominal viscera for approximately thirty minutes. The rats were allowed to recover from the anesthetic, and were assayed 18 hours later. During the recovery period the rats did not eat, and at the time of assay a 57% loss in hepatic Δ^4-steroid hydrogenase activity was found. However, if animals similarly treated were force-fed a liquid diet in the same way as described for the group at the right of Figure 7, this effect of surgical trauma on the enzymatic activity was completely prevented. Nevertheless, these animals still showed an adrenal cortical

PLASMA CORTICOSTERONE CONCENTRATION (uncorrected for biological blank) µg/100 ml plasma	8.4 ± 1.01 (7)			22.4 ± 4.6 (8)	2.18 ± 0.3 (6)	2.56 ± 0.5 (6)
ADRENAL WEIGHT mg / 100 mg BW	15.8 ± 0.4 (12)	17.8 ± 1.6 (6)	17.1 ± 0.4 (5)	17.5 ± 1.08 (8)	5.52 ± 0.2 (6)	5.23 ± 0.28 (6)

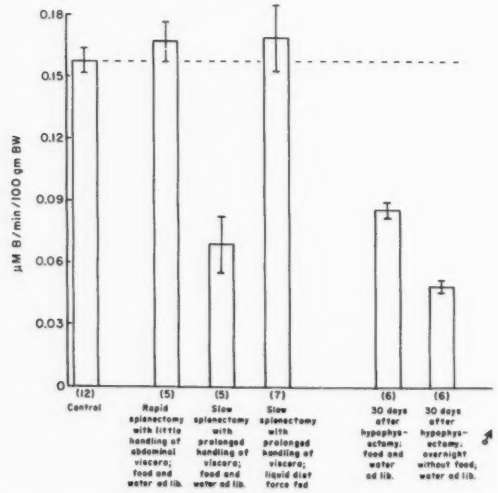
µM B/min/100 gm BW
0.18
0.15
0.12
0.09
0.06
0.03
0
(12) Control
(5) Rapid splenectomy with little handling of abdominal viscera; food and water ad lib.
(5) Slow splenectomy with prolonged handling of viscera; food and water ad lib.
(7) Slow splenectomy with prolonged handling of viscera; liquid diet force fed
(6) 30 days after hypophysectomy; food and water ad lib.
(6) 30 days after hypophysectomy; overnight without food, water ad lib.

FIG. 8. Prevention, by force-feeding, of the impairment in corticosterone metabolism in liver slices which follows surgical trauma; independence of the impairment in steroid metabolism following noxious stimuli from changes in either pituitary or adrenal cortical function.

response to trauma, as indicated by the nearly three-fold increase in plasma corticosterone concentration at the time of assay (top line, Fig. 8). The results did not depend in any way upon the presence or absence of the spleen itself, as shown by the second group from the left in Figure 8. In this group the spleen was removed rapidly and gently. Food intake was not affected, and the hepatic Δ^4-steroid hydrogenase activity was normal.

In the second experiment the effects of an overnight fast on the enzymatic activity of livers from hypophysectomized animals were studied thirty days after hypophysectomy. Three criteria were employed to test the adequacy of the hypophysectomy: a) the uncorrected plasma corticosterone concentrations were very low—at the level of the plasma blank observed in adrenalectomized rats, b) the adrenals were severely atrophic, and c) the sella turcica regions were devoid of pituitary remnants on gross inspection.

The hypophysectomized animals allowed food and water had much lower enzymatic activities than did normal controls (Fig. 8), as previously noted by Forchielli and Dorfman (18). Nevertheless, overnight fast in hypophysectomized animals caused a 43% decrease in hepatic Δ^4-steroid hydrogenase activity from the already low level observed in the fed hypophysectomized group. No increase in plasma "corticosterone" concentration occurred.

Effect of loss of hepatic Δ^4-steroid hydrogenase activity on plasma corticosterone concentration

From the prolongation of the *in vivo* half-life of corticosterone in the plasma of fasted female rats (Fig. 6) we concluded that impairment of ring A reduction of adrenal cortical hormones could contribute to the rise in plasma concentration of corticosteroids observed in animals exposed to noxious stimuli. We tested this conclusion more directly in two groups of male rats. The control group was allowed food and water *ad libitum* and the experimental group was fasted overnight in the usual fashion, to diminish the hepatic Δ^4-steroid hydrogenase activity by approximately 50% (Figs. 3, 5 and 7). Animals in each group received either 5 μg./100 gm. B.W. of corticosterone in saline with a trace of ethyl alcohol or an equal volume of saline-alcohol alone intravenously, ten minutes after injection of 5 mg./100 gm. B.W. of pentobarbital I.P. Fifteen minutes after the intravenous injections aortic blood was obtained for analysis. The results are shown in Figure 9.

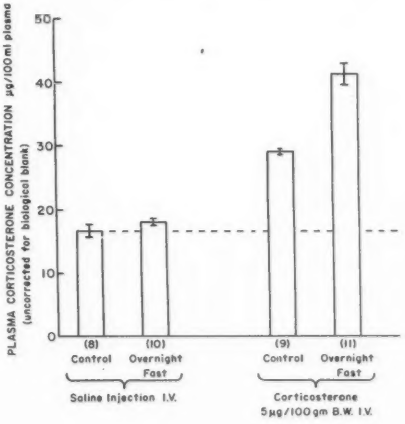

FIG. 9. Effects of overnight fast on the plasma corticosterone concentration following intravenous injection of a physiological dose of corticosterone.

The intravenous injection of corticosterone caused an increase in plasma corticosterone concentration of 12.5 μg./100 ml. in the control, fed animals. In the fasted animals the same dose of corticosterone caused an increment in plasma corticosterone concentration nearly twice as great (23.1 μg./100

ml.). All the animals weighed about 100 grams, and the extracellular fluid volume into which corticosterone might be expected to distribute in the time allowed was presumably 20 to 25 ml. The maximum increment of corticosterone concentration possible in this volume following injection of 5 μg. of corticosterone (assuming complete mixing)—if there were no metabolism of the steroid at all—would be 20 to 25 μg./100 ml. Since this was the magnitude of the observed increment in fasted animals, it appears that the impairment of the *in vivo* inactivation of corticosterone, indicated by the results shown in Figure 9 and predicted from the *in vitro* experiments, must have been severe.

DISCUSSION

Previous *in vitro* studies of the metabolism of adrenal cortical hormones by the livers of animals exposed to noxious stimuli are few. Paschkis *et al.* have reported that, insofar as could be determined by their bioassay techniques, slices and homogenates of various tissues, including liver, taken from rats exposed to formaldehyde, inactivated cortisone to the same extent as did tissues from control rats (23). Zacco *et al.* reported that the rates of reduction by rat liver of the Δ^4-3-keto groups, or the C_{20} carbonyl group of cortisol and cortisone were severely diminished in animals fasted 72 hours and exposed to cold for four hours, compared to those observed in control animals fasted overnight only. They concluded that the "rates of metabolism of compounds E and F . . . (are) definitely lower in stressed than in normal animals" (24).

Our results demonstrate that noxious stimuli which disturb the food intake of rats also impair hepatic inactivation of corticosterone to a degree which is closely related to the change in food consumption. Variations in water intake and in pituitary or adrenal cortical function are not involved in this relationship. Conversely, in these experiments stimuli which failed to diminish food intake appreciably (croton oil granuloma pouch, swimming, repeated ether anesthetization immediately before assay, exposure to cold, repeated handling and forced-feeding of a liquid diet) failed also to affect the inactivation of corticosterone by ring A reduction. Thus the experimental "stress" employed by Zacco *et al.* appears to have been fortunately chosen for their purposes, since it involved a change in food intake. Presumably the "stress" examined by Paschkis *et al.* did not.

Huseby *et al.* in an excellent study in men demonstrated that inanition without severe water restriction caused a reduction in urinary excretion of 17-hydroxy corticosteroids, while the levels of such compounds in the blood remained normal or even were elevated (25). They suggested that, among other possibilities, an overall slowing of corticosteroid metabolism might explain their results. Our experiments lend further strength to that interpretation.

The supply of TPNH from the hexose-monophosphate shunt has been

shown to be profoundly altered under some conditions of fasting and refeeding (26, 27). It seems probable that a non-specific diminution in TPNH supply within liver cells was responsible for the loss of Δ^4-steroid hydrogenase activity per gram of liver in the slice assay, since the activity per gram of liver in the homogenate assay, in which exogenous TPNH is abundant, was not affected by reductions in food intake.

Although in our experiments all the changes in steroid metabolism could be accounted for by changes in food intake, nevertheless, the decreased corticosteroid metabolism believed to occur in man and dogs after very acute noxious stimuli (6, 5) may be brought about in part by reduction in hepatic blood flow. But at present there is no evidence which proves that steroid metabolism is ever blood-flow limited. To prove the existence of such a limit it would be necessary to show that the liver cannot increase the extraction of adrenal cortical hormones (i.e. the portal vein—hepatic vein corticosteroid concentration difference) under conditions of reduced flow. If reductions in flow are accompanied by increased extraction, as is the case with the oxygen supply to skeletal muscle, then stéroid metabolism by liver might be very insensitive to changes in hepatic blood flow over a wide range of flows.

In the course of the experiments reported here we obtained three lines of evidence that variations in the rate of ring A reduction of adrenal cortical hormones by liver tissue *in vitro* accurately predict variations in the overall rate of corticosteroid metabolism in intact animals: a) livers from females have greater Δ^4-steroid hydrogenase activity *in vitro* than have those from males, and the half-life of corticosterone *in vivo* is significantly less in the female; b) overnight fast causes extensive loss of Δ^4-steroid hydrogenase activity from liver *in vitro*, and prolongs the half-life of corticosterone *in vivo;* and c) the loss of hepatic capacity to reduce ring A of adrenal cortical hormones *in vitro* after overnight fast is associated with a greater than normal elevation of plasma corticosteroid concentration *in vivo* after a given increase in corticosteroid inflow. But even though impairment of hepatic reduction of ring A of adrenal cortical hormones does lead to greater than normal elevations of plasma corticosteroid concentration following a given intravenous infusion of the hormones, it probably does not have such an effect during the response of animals to noxious stimuli. As long as the negative feedback control of adrenal cortical secretion rate is operative, impairment of hepatic inactivation of adrenal cortical hormones will merely diminish the increment of adrenal cortical secretion rate required to achieve a new high plasma corticosteroid concentration, and will not affect the concentration itself.

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CORTICOSTEROIDOGENIC POTENTIAL OF REGENERATED RAT ADRENAL AUTOGRAFTS AND ENUCLEATED GLANDS[1]

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ABSTRACT

The corticosteroidogenic potential of regenerated rat adrenal autografts to mesenteric and intramuscular sites and of enucleated glands regenerated *in situ* was measured *in vitro* by blue tetrazolium and ultraviolet-analysis of methylene dichloride extract residues of Krebs-Ringer incubation medium and *in vivo* by the water intoxication test. Compared to intact controls, adrenals regenerated 30 days or more exhibited normal *in vitro* output rates/unit tissue weight in the absence or presence of ACTHar (10 I.U./100 mg. adrenal) irrespective of regeneration site or age. Mesenteric transplants regenerated 14 days showed approximately 50% reduction in output rates without apparent loss of ACTH sensitivity. None afforded normal protection against water intoxication after regenerating 30, 60 or 90 days. *In vivo*, enucleated glands were functionally superior to transplants at all periods, while the mesenteric were superior at 60 but similar at 90 days to intramuscular transplants. Relative functional ability was generally but not consistently correlated with corresponding adrenal weight differences.

IT IS well established that adrenocortical regeneration occurs following autoplastic transplantation or enucleation of rat adrenals, but it is probable that regenerated glands are never able to respond functionally to stress in a normal way (1). Previous studies from this laboratory have shown that the normal protective capacity of adrenocortical tissue in situations requiring rapid mobilization of steroidal secretion is impaired following autoplastic transplantation to intramuscular and other tissue sites (2). Resumption of function, albeit subnormal, is well established by the 4th day of regeneration with maximal possible protection occurring approximately by the 30th day (3, 4). Eversole, Edelmann and Gaunt (5) have shown that adrenocortical autografts made to six different sites vary in functional efficiency and that none affords normal protection against water intoxication after 42–50 days of regeneration. Of the sites tested, grafts to the ovary, kidney and intestinal mesentery were all approximately of equal functional efficiency and were, moreover, superior to those made to the liver, eye, or muscle. Enucleated glands allowed to regenerate *in situ* were more effective than any of the transplants.

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These findings suggest as possible explanation of impaired functional efficiency quantitative and/or qualitative alterations in hormone biosynthesis, reduction in corticoid release related to impaired glandular circulation or to relative amounts of tissue regenerated, reduction in sensitivity to adrenocorticotropin (ACTH), or perhaps a combination of these and other factors. In fact, Houssay (6) has suggested that partial protection afforded by regenerated enucleated glands against histamine intoxication may be explained on the basis of reduced sensitivity to ACTH. Quantitative and qualitative *in vivo* and *in vitro* findings of corticoid secretion in regenerated rat adrenals have been equivocal thus far (1). However, one recent *in vitro* study (7) indicates that, compared to intact glands, enucleated adrenals regenerated *in situ* in uninephrectomized, uniadrenalectomized young albino rats produce lowered quantities of blue tetrazolium (BT) reducing substance at 21 days but regain normal secretory capacity at 30 days. Furthermore, they remain responsive to ACTH and do not show any significant qualitative imbalance in output, although both aldosterone and corticosterone are secreted in subnormal amounts.

This is the first report in a series of investigations to evaluate systematically by direct biochemical methods the *in vitro* corticosteroidogenic potential of adrenocortical autotransplants regenerating in otherwise undisturbed hosts. Specifically, the present studies were designed to provide quantitative biochemical evidence of adrenal cortical hormone production as related to transplantation site, period of regeneration, and ability to provide protection against water intoxication.

METHODS

General

Autoplastic transplantation of adrenocortical tissue was performed under ether anaesthesia in Wistar or Sprague-Dawley CD[2] (caesarean delivered) male albino rats weighing 100–120 gm. each. Transplants (two halves of one gland) were made either to the dorsal musculature by established methods (cf. 3) or to the intestinal mesentery by tying two halves of the left adrenal, strung on a suture, to adjacent positions on the mesenteric vein, followed by removal of the right adrenal. Enucleation was performed simultaneously on both adrenals and the glands allowed to regenerate *in situ*. All animals were given 1% saline drinking water for the first 7 days after operation and then maintained on tap water and Purina chow *ad libitum*. Glands were allowed to regenerate for a variable number of days. Controls with intact adrenals similar in every other respect to operated animals were provided for each group. Intact or regenerated adrenals were removed under sodium pentobarbital anaesthesia, trimmed of periadrenal tissue, halved, weighed on a torsion micro-balance, and distributed to paired vessels containing 2.0 ml. ice-cold Krebs-Ringer-phosphate-glucose medium (pH 7.1) so that each pair received matched tissue at least equivalent in weight to one intact gland. Prior to weighing, as much as possible of the calcified core occurring typically in regenerated glands was removed without excessive injury to viable tissue. Incubation was carried out in an atmosphere of pure oxygen at 38° C in a Warburg apparatus according to methods pre-

[2] Obtained from Charles River Breeding Laboratories, Brookline, Mass.

viously described (8). After pre-incubation for 30 minutes, incubation for 2 hours was carried out in fresh medium with ACTH (Armour ACTHar) in doses of 10,000 mU./100 mg. adrenal added to one flask of each pair containing matched tissue. The incubation fluid was extracted with spectroscopically pure methylene dichloride (Merck) (9, 10). An 80% aliquot of the solvent phase was evaporated to dryness at 60° C under nitrogen and desiccated *in vacuo* for 30 minutes over Drierite. Dried extracts were dissolved in 1.0 ml. reagent grade absolute ethanol (twice redistilled) and duplicate measurements of corticosteroids made directly on aliquots of the alcoholic solution by the blue tetrazolium (BT) micromethod (11) and by measurement of peak absorption in the ultraviolet at 240 mμ (12) using a Beckman Model DU Spectrophotometer. These determinations were performed against 2.0 ml. aliquots of incubation medium "blank" carried through the entire procedure. Addition of ACTH to the medium "blank" did not significantly change the BT or UV optical densities. Output rates have been calculated as μg. corticosterone equivalents/100 mg. adrenal tissue (wet weight)/incubation time. Corrections were not made for medullary tissue in intact adrenals, since in the male albino rat it comprises only 5–6% of the total adrenal weight (13).

The water intoxication test was performed according to methods previously described (3). After testing, adrenals were recovered and weighed individually as described above. Animals with gross evidence of accessory adrenal tissue were discarded.

Corticosteroid recovery with methylene dichloride. We have investigated systematically recovery of crystalline corticosterone added to 2.0 ml. aliquots of incubation medium in concentrations anticipated in tissue experiments (1–10 μg./ml. incubation medium) and carried through the same procedures of incubation, extraction, and quantification. Recoveries generally in excess of 90% were obtained for all concentrations tested with fairly good correlations between BT and UV methods. Our level of recovery is in general agreement with that reported by Rosenkrantz (10) using methylene dichloride as the extraction solvent.

Effect of bicarbonate vs phosphate medium on adrenal sensitivity to ACTH. The use of bicarbonate-buffered medium in the *in vitro* investigation of adrenocortical secretion has largely superseded the use of phosphate-buffered medium, since at physiological pH calcium remains more readily in solution in the former. Calcium appears to be necessary for efficient ACTH action *in vitro* (14). However, phosphate-buffered medium is more easily handled routinely and calcium remains in solution well at pH near 7.1. Since we have used this system in the present investigation, response to various small doses of ACTH by intact adrenocortical tissue incubated in bicarbonate (pH 7.4) and phosphate (pH 7.1) buffered media has been compared. Our findings show that per cent increase in adrenal output in the presence of ACTH is essentially similar in the two systems at dose levels of 20, 100, and 500 mU./100 mg. adrenal. Further, these data, in agreement with other observations, show that the minimal dose necessary for maximal response of intact rat adrenals in phosphate-buffered Krebs-Ringer medium lies between 100 and 500 mU./100 mg. adrenal (8). Thus, the ACTH dose used in our regenerated tissue series is at least 20× the minimal dose required for maximal response with intact adrenals.

Effect of preincubation on ACTH sensitivity. Preincubation of isolated intact rat adrenals before incubation in fresh medium containing ACTH greatly increases the sensitivity of the glands to ACTH stimulation (8). We found that this applies as well to regenerated adrenocortical tissue. Intramuscular or mesenteric autotransplants, or adrenals enucleated *in situ*, allowed to regenerate more than 30 days (output rates/unit tissue weight are approximately normal after 30 days, *v.i.*), and intact control glands were preincubated before addition of ACTH as described above in one series, and incubated for two and one-half hours without change of medium (ACTH added to one flask of each pair after one-half hour) in another. In both output was measured with the

BT method. Comparison of results from the two series showed that preincubation increases the sensitivity to ACTH of regenerated as well as of intact adrenals from approximately two to six times.

RESULTS AND DISCUSSION

Effect of transplant site on growth and output

Greater regeneration has been obtained from mesenteric than from intramuscular adrenal autografts in the rat (5). Since the largest possible amount of tissue per regenerating transplant was needed for biochemical analysis of output, comparison was made of the growth and function of autografts to mesenteric, subcutaneous (ear), and intramuscular sites and of enucleated glands regenerated *in situ* in Wistar rats. At 30 days of regeneration, transplants, irrespective of site, appear similar histologically and in ability to maintain life. Average weights of mesenteric autografts are greater than those of other transplants studied, but less than enucleated glands (Table 1). Further, the sequence of cell types reported for intramuscular transplants (15) as determined by sudanophilic lipid content occurs in the same order in enucleated glands and mesenteric transplants, but is advanced by 2–7 days (16).

In vitro output of Wistar rat autografts to mesenteric and intramuscular sites and of enucleated glands regenerated 30 days was compared to that of intact controls in the absence and presence of ACTH. Subcutaneous transplants were not tested since adequate quantities of tissue could not be obtained consistently. Our findings (Table 2) show good correlation between BT and UV values despite differences in magnitude. Furthermore, average output rates of regenerated glands irrespective of regeneration site are approximately similar in magnitude to those of intact controls in the absence of ACTH. Following ACTH stimulation, all transplants show average increases in output rate and ACTH sensitivity comparable to those of intact controls (except as measured by UV). Although these data suggest that intact glands are somewhat more sensitive to ACTH stimulation than

TABLE 1. EFFECT OF TRANSPLANTATION SITE ON ADRENAL WEIGHT OF WISTAR MALE ALBINO RATS

Adrenal preparation	No. glands	Transplant age (days)	Total adrenal wt. (mg.)
Intact	7	—	38.0 ±4.06*
Subcutaneous (ear) autograft	7	21	9.92±4.84
	12	210	12.0 ±3.85
Intramuscular autograft	19	30–32	13.1 ±3.78
Mesenteric autograft	10	30	18.1 ±6.57
Regenerated enucleated gland	12	33–36	26.6 ±7.41

* Average ± standard deviation.

TABLE 2. *In vitro* OUTPUT RATES IN THE ABSENCE AND PRESENCE OF ACTH OF MALE ALBINO WISTAR RAT ADRENALS REGENERATED 30 DAYS IN VARIOUS TRANSPLANTATION SITES

Adrenal preparation	No. experiments	Adrenal output (μg./100 mg. adrenal/2 hrs. incubation)							
		BT				UV			
		No ACTH	ACTH	Net increase	% increase	No ACTH	ACTH	Net increase	% increase
Intact	7	15.6 ± 9.85*	60.1 ± 9.98	44.5 ± 10.4	285	16.2 ± 6.00	76.9 ± 9.87	60.7 ± 7.41	375
Intramuscular autograft	4	13.9 ± 2.76	45.3 ± 10.6	31.4 ± 10.3	226	12.4 ± 2.14	47.7 ± 17.2	35.3 ± 15.9	284
Mesenteric autograft	2	16.8 ± 1.53	57.8 ± 5.94	41.0 ± 7.17	244	13.2 ± 0.95	52.4 ± 8.35	39.2 ± 7.45	299
Regenerated enucleated gland	5	17.0 ± 6.11	52.6 ± 12.2	35.7 ± 9.76	210	15.9 ± 7.18	47.0 ± 12.7	31.1 ± 5.73	196

* Average ± standard deviation.

TABLE 3. *In vitro* OUTPUT RATES IN THE ABSENCE AND PRESENCE OF ACTH OF MALE ALBINO SPRAGUE-DAWLEY (CD) RAT ADRENAL AUTOGRAFTS REGENERATED IN EXCESS OF 30 DAYS IN THE MESENTERIC SITE

Regeneration (days)	Adrenal preparation	Total adrenal wt. (mg.)	Adrenal output (μg./100 mg. adrenal/2 hrs. incubation)*								Net increase ratio: BT/UV
			BT				UV				
			No ACTH	ACTH	Net increase	% increase	No ACTH	ACTH	Net increase	% increase	
30	Intact	39.8 ± 6.65† (6)	14.7 ± 6.10 (3)	39.1 ± 7.20	24.4 ± 12.4	167	19.5 ± 9.40	50.8 ± 0.83	31.3 ± 8.70	161	0.78
	Transplant	20.0 ± 8.90 (26)	14.2 ± 4.40 (7)	34.8 ± 7.40	20.6 ± 7.10	145	14.0 ± 8.40	44.3 ± 7.70	30.3 ± 6.80	216	0.68
60	Intact	39.0 ± 4.75 (4)	12.2 ± 2.60 (2)	36.5 ± 5.00	24.3 ± 2.40	199	9.30 ± 3.70	50.2 ± 9.80	40.9 ± 13.5	439	0.60
	Transplant	27.0 ± 13.5 (14)	9.10 ± 2.00 (5)	30.6 ± 6.20	21.5 ± 7.90	236	8.60 ± 2.80	40.5 ± 7.00	31.9 ± 7.96	371	0.67
90	Intact	48.0 ± 1.41 (4)	17.3 ± 8.00 (3)	49.8 ± 22.4	32.5 ± 22.6	188	20.3 ± 5.40	62.9 ± 31.4	42.6 ± 30.1	210	0.76
	Transplant	36.9 ± 12.9 (15)	17.2 ± 4.80 (7)	42.9 ± 11.0	25.7 ± 8.20	150	19.3 ± 5.00	55.7 ± 13.3	36.4 ± 10.8	189	0.71

* Average ± standard deviation in each series for number of experiments indicated in parentheses in first column.
† Average ± standard deviation for number of glands indicated in parentheses.

transplants, these results were not obtained consistently enough in further studies with mesenteric transplants (cf. Table 3) to draw valid conclusions.

Effect of regeneration age on adrenal output

Mesenteric autografts were further studied because of superior tissue yields and similarity in corticosteroidogenic potential with other 30-day regenerated preparations. Because of wide variations in survival numbers among various lots of Wistar rats following transplantation procedures, Sprague-Dawley CD rats were employed predominantly in the following investigations.

Output *in vitro* was determined at 14, 30, 60 and 90 days of regeneration and compared to that of appropriate intact controls. Compared to controls, transplants regenerated 30, 60 and 90 days do not show marked differences in output rates/unit weight in the absence or presence of large ACTH doses or consistent differences in ACTH sensitivity (Table 3). However, transplants regenerated 14 days, whether from Wistar or Sprague-Dawley rats, exhibit approximately 50% reduction in output rates compared to intact controls, but without a significantly uniform alteration of ACTH sensitivity (Table 4). These latter findings are generally in agreement with similar observations of Masson, Koritz and Peron (7) for enucleated rat adrenals regenerating *in situ* 21 days. Apparent reduction in output rate in the 14-day series may arise from failure to account for nonviable tissue which comprises about 50% of the total adrenal weight in 14-day mesenteric transplants (16) or may actually reflect quantitative changes in corticoid secretion. Consistent BT/UV ratios computed from output due to ACTH stimulation in glands regenerated in excess of 30 days parallel those of respective intact controls and are, on the whole, similar to those reported for intact adrenals (17). In contrast, inconsistent variations were observed in BT/UV ratios in 14-day regenerated transplants. The data also show progressive increase in regenerated tissue weight with time.

Effect of adrenal weight and regeneration age on response to water intoxication

Our data show that regardless of site or age of regeneration, adrenals regenerated in excess of 30 days exhibit approximately normal output rate/unit weight and response to large ACTH doses. However, relative differences in total adrenal weight related to age and site of regeneration suggested strongly that ability to protect against systemic stress might be a function of hormonal production related to quantities of steroidogenically active tissue rather than to quantitative alterations in corticosteroidogenic potential.

This hypothesis was tested quantitatively by means of the water intoxication test. Wistar rats were used since output rates of glands regenerating for 30 days in various sites had been determined previously. Mesenteric

TABLE 4. COMPARISON OF OUTPUT RATES IN ABSENCE AND PRESENCE OF ACTH OF 11-DAY REGENERATING MESENTERIC AUTOGRAFTS IN WISTAR AND SPRAGUE-DAWLEY (CD) MALE ALBINO RATS

Rat	Adrenal preparation	Total adrenal wt. (mg.)	Adrenal output (μg./100 mg. adrenal/2 hrs. incubation)*								Net increase ratio: BT/UV
			BT				UV				
			No ACTH	ACTH	Net increase	% increase	No ACTH	ACTH	Net increase	% increase	
Wistar	Intact	45.0±3.00† (2)	19.9 ±1.15 (2)	57.7±23.2	37.8±24.3	190	14.9 ±1.34	80.7±22.1	65.8±20.9	441	0.58
	Transplant	14.0±3.59 (9)	7.63±0.90 (3)	30.3± 1.83	22.7± 5.44	299	9.10±0.95	23.7± 9.96	14.6± 3.10	160	1.55
Sprague-Dawley	Intact	34.3±1.79 (4)	17.0 ±7.10 (4)	66.9±13.4	49.8±18.0	292	23.8 ±6.30	88.0±17.2	65.0±23.4	274	0.77
	Transplant	10.8±3.93 (21)	7.60±3.30 (4)	26.4±10.7	18.8± 9.60	248	7.30±2.20	45.4± 9.80	38.2±12.0	523	0.49

* Average ± standard deviation in each series for number of experiments indicated in parentheses of first column.
† Average ± standard deviation for number of glands indicated in parentheses.

TABLE 5. RELATIONSHIP BETWEEN GLANDULAR WEIGHT AND RESPONSE TO WATER INTOXICATION OF WISTAR MALE ALBINO RATS WITH ADRENAL AUTOGRAFTS REGENERATING IN VARIOUS SITES

Regeneration (days)	Adrenal preparation	H_2O* excreted			Total adrenal wt.	
		No. rats	%	% of intact excretion H_2O	No. adrenals	mg.
30	Intact	9	96.0± 3.4†	100	9	42.7± 5.6†
	Enucleated	9	86.7±14.3	90	9	26.9± 6.2
	Mesenteric	9	66.5±29.3	69	9	26.2± 8.4
	Intramuscular	5	40.8±16.7	43	5	9.60± 3.4
60	Intact	9	95.2± 4.6	100	9	49.7± 8.0
	Enucleated	9	91.0± 4.3	96	9	22.3±12.2
	Mesenteric	7	86.5± 7.2	91	7	18.0± 6.0
	Intramuscular	7	23.2±15.8	24	7	16.7± 5.3
90	Intact	8	94.8± 5.1	100	6	39.9± 5.4
	Enucleated	9	90.7± 8.3	96	6	33.6± 4.7
	Mesenteric	9	85.9± 5.1	91	6	19.7± 4.6
	Intramuscular	7	84.6± 8.4	89	4	19.7±10.6

* Water administered by gavage equal to 24% body weight.
† Average ± standard deviation.

and intramuscular autografts and enucleated glands regenerated for 30, 60, and 90 days were compared with appropriate intact controls. None of the regenerated glands affords normal protection within the 90-day regeneration period (Table 5). Enucleated glands appear to be functionally superior to transplants at all periods tested, while mesenteric transplants are superior at 60 days but similar at 90 days to intramuscular transplants. These findings on the relative protection afforded by intact and regenerated glands are in general agreement with previous findings (2, 5). Moreover, our data show that enucleated glands and mesenteric transplants reach peak performance in 60 days whereas intramuscular transplants do not. These data show that functional superiority is generally although not consistently correlated with corresponding regenerated tissue weight superiority.

Our present findings indicate that an explanation of reduced ability to protect against systemic stress evident in adrenals regenerated for 30 days must reside in factors other than those related to major quantitative alterations in hormone production or exclusively to amount of regenerated tissue. Inavailability of medullary hormone may be a factor. Demonstration by us that regenerated adrenocortical tissue regardless of site or age of regeneration in general remains normally responsive to large ACTH doses does not preclude the possibility of reduced response at more physiological concentrations. Preliminary findings by us suggest this as a strong possibility. Furthermore, differences in extent or mode of revascularization associated with transplant site might result in abnormal transport of corticosteroid precursor or of ACTH to, or of corticoids from, regenerating glands. Qualitative changes in corticoid secretion might serve as an additional explanation. Demonstration by us and others of quantitative reduction in output prior to 30 days regeneration might reflect qualitative changes in secretion persisting beyond this period. Recent findings (7) indicating no major qualitative imbalance in steroidal secretion of enucleated glands regenerated *in situ* 21 and 30 days do not entirely exclude this latter possibility, since this might not apply to glands regenerating under less abnormal conditions, or since qualitative differences in secretion which might occur could remain undetectable by current methods of analysis. The significance of these latter possibilities merits further investigation.

Acknowledgments

We wish to express our gratitude to our research associate, Miss Patricia Griffin, and to Mr. Foster Burnett and Mrs. Lois Drapeau for skillful technical assistance.

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SITE OF ORIGIN OF A HYPERGLYCEMIC SUBSTANCE LIBERATED INTO THE PANCREATO-DUODENAL BLOOD BY DESERPIDINE INJECTIONS[1]

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ABSTRACT

The injection of deserpidine into the pancreato-duodenal artery of normal dogs is followed by a prompt rise in blood sugar concentration. This hyperglycemia is not observed in previously depancreatized dogs. The results suggest that deserpidine causes the release of a hyperglycemic substance originating in the pancreas. The nature of this substance is unknown, although it appears to be different from the hyperglycemic material released by the injection of growth hormone.

A HYPERGLYCEMIC substance or substances have been found by several investigators in the blood of the pancreato-duodenal vein following different stimuli such as the injection of insulin, growth hormone or deserpidine (1–5). These substances have not been identified, but can be described as follows: 1) a material released during insulin hypoglycemia, which resembles glucagon (6) and may be similar to that found in normal blood (3, 7, 8); 2) a material released by growth hormone, which is believed to be a sympathomimetic amine similar to that found in normal urine (9) primarily because its action, contrary to that of glucagon (5, 10), is inhibited by dihydroergotamine (DHE) (11); this amine could be epinephrine or serotonin (12), either of which is capable of raising blood glucose (5, 13–16); and 3) a material released by deserpidine which appears only when the drug is injected directly into the pancreato-duodenal artery of normal dogs and not when the pancreas is bypassed (5). Although deserpidine releases serotonin and the catecholamines from their storage sites (17), the action of this material is not prevented by blocking agents such as DHE or SA-97 (5, 18). These observations suggest that the pancreato-duodenal area produces at least two types of hyperglycemic substances: one type released by growth hormone which, like serotonin and the catecholamines, is blocked by DHE, and another type, released by in-

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ection of deserpidine which, like glucagon, is not affected by this blocking agent. The purpose of this work was to investigate whether the hyperglycemic material liberated by deserpidine originates in the pancreas, like glucagon, or in the duodenum, like serotonin.

MATERIALS AND METHODS

Nine mongrel dogs of both sexes were depancreatized under barbiturate anesthesia (pentobarbital sodium, 35 mg./kg. and barbital sodium, 100 mg./kg., intravenously). Special care was taken not to injure the pancreato-duodenal artery and vein and their major duodenal branches. At the end of the operation, the animals received 100 cc. of 10% glucose in saline and 1 cc. of a depot antibiotic, subcutaneously. On the days following the operation the dogs were offered a stock diet of commercial dog food, raw horse meat and pancreatin and, as their appetite returned, were given increasing doses of regular insulin. Occasionally, if the appetite was poor, subcutaneous administration of glucose in saline was repeated to prevent hypoglycemia. Antibiotics were continued until the abdominal wound had healed satisfactorily. At this time, insulin was withdrawn and ketonuria allowed to develop; this was considered tentative evidence of satisfactory removal of the pancreas until final confirmation at autopsy. After ketosis had developed, the animals were again given regular insulin in doses sufficient to reduce the 24-hour glucose excretion to a trace and kept on this management for 3 to 20 days, until they appeared healthy and well nourished. When convenient, the dogs were re-anesthetized as stated above, the duodenal (formerly pancreato-duodenal) artery was carefully dissected from the post-operative adhesions and cannulated as described elsewhere (19). After the preparation was completed, the abdominal incision was approximated with hemostats and the animal allowed to rest for at least one hour so that any effects of surgical trauma may disappear. Three blood samples were then taken at 15 minute intervals to make sure that the blood sugar had reached normal and stable values. After this, deserpidine[3] (1 mg./ml./kg., dissolved in acetic acid at pH 3.5) was injected slowly into the duodenal artery. Blanching of a large portion of the duodenum and return of the normal color immediately after the injection demonstrated effective perfusion of the organ and adequate arterial blood supply bypassing the obstruction at the site of cannulation. Glucose was determined in duplicate blood samples, according to Nelson (20). The results were compared statistically (21) to those obtained following deserpidine injections into a mesenteric or into the pancreato-duodenal artery of normal dogs (5).

RESULTS AND DISCUSSION

Figure 1 shows the average changes in blood glucose caused by deserpidine. Injections into the duodenal artery of depancreatized dogs cause only a small and delayed rise in the concentration of blood glucose. This rise, like the rise caused by injections of deserpidine into a mesenteric artery of normal dogs, is not statistically significant and differs sharply from the marked and significant hyperglycemia observed following injections into the pancreato-duodenal artery of normal dogs. These results suggest that the rapidly active hyperglycemic material responsible for this marked rise originates in the pancreas. Although the identity of this material remains

[3] Gift of Abbott Laboratories. Deserpidine is 11-desmethoxyreserpine, an alkaloid derived from the root of *Rauwolfia canescens*.

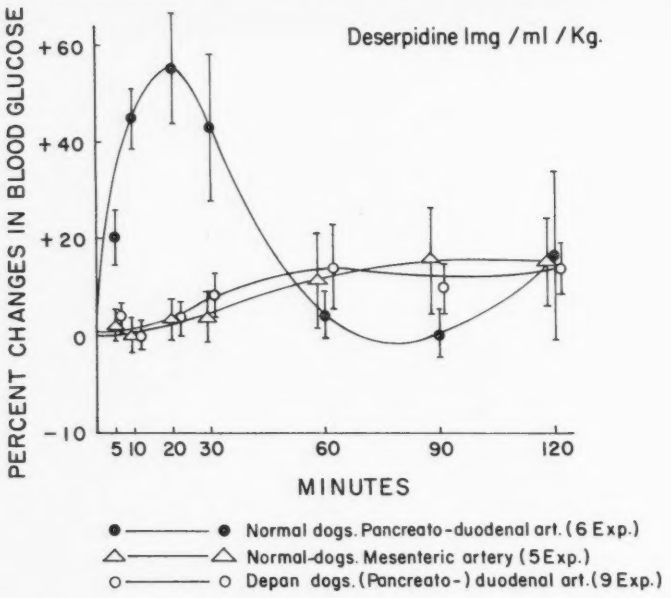

FIG. 1. Effect of deserpidine on blood glucose after injection into the pancreato-duodenal or into a mesenteric artery of normal dogs (5) and after injection into the duodenal artery of depancreatized dogs. Initial concentrations: normal dogs, 60 to 97 (ave. 84 mg. %); diabetic dogs, 120 to 282 (ave. 198 mg. %).

uncertain, because of its resistance to the blocking action of DHE and SA-97, it resembles glucagon and differs from the material released by growth hormone. The latter material is blocked by DHE, persists after the removal of the pancreas (11) and, therefore, resembles serotonin which is also blocked by DHE and is formed primarily in the mucosa of the duodenum and upper jejunum (22, 23).

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UNCOUPLING OF THE OXIDATIVE PHOSPHORYLATION WITH CORTISONE IN LIVER MITOCHONDRIA[1]

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ABSTRACT

Oxidative phosphorylation was determined in the liver mitochondria of control rats and in young, middle-aged and old rats of both sexes treated for 7 days with 5 mg. cortisone acetate. Isocitric, α-ketoglutaric, succinic, malic and pyruvic acid were used as substrates. A total of 172 rats were used. The technique is described in detail. When the substrate was isocitrate or α-ketoglutarate, oxygen consumption was significantly decreased in old female rats. The phosphorus utilization obtained with the same acids declined in most cases, most strongly in old female animals. With succinate, malate and pyruvate the utilization of phosphorus in male rats was variably inhibited. In female rats there was a significant decrease only in old animals, and it was mostly highly significant. The P/O ratio was reduced in young female and old male rats, particularly in old females. It may be concluded that cortisone frequently inhibits oxidative phosphorylation in liver mitochondria of rats of all ages and both sexes, most strongly in females over 12 months old. Estradiol, injected simultaneously with the cortisone into old female rats, counteracts the uncoupling effect of cortisone. Growth hormone, androgenic hormone and testosterone produced no effect. Uncoupling of the oxidative phosphorylation by cortisone and inhibition of the associated ATP production seems to provide an explanation for many of the tissue changes and clinical symptoms caused by cortisone.

DESPITE extensive research work, the mechanism of the cortisone effect is as yet largely unexplained. It is known, however, that cortisone influences both the carbohydrate, fat and protein metabolism. The changes produced in the carbohydrate metabolism are best known. A number of investigators have demonstrated that cortisone may produce a negative nitrogen balance. Administered in large doses, cortisone causes a great increase in urinary excretion of nitrogen (1). It is not yet known whether cortisone inhibits the protein synthesis or whether it stimulates protein catabolism. Current opinion seems to favour the latter theory. Using the isotope technique, Derache *et al.* (2) studied the effect of large doses of cortisone on ATP synthesis in the liver and found that it was greatly accelerated. Clark *et al.* (3) are of the opinion that cortisone reduces the oxygen consumption in the mitochondria of rat liver but that the P/O ratio remains unchanged. Their experiments were carried out on 11 young female rats, using α-ketoglutarate as substrate and giving daily

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injections of 2.5 mg. of cortisone per 100 gm. of body weight during 12–13 days.

MATERIAL AND METHOD

In the following experiments the consumption of oxygen and of phosphorus per mg of nitrogen were determined in rat liver mitochondria, which contain the components of the Krebs cycle. The test animals—rats of Sprague-Drawley strain—were divided into three age groups, i.e. young (2–4 months), middle-aged (6–8 months), and old rats (12-16 months). The sex incidence was approximately equal. A total of 172 rats were used. They were kept on an unrestricted, mixed diet. About one-half of the animals were given a subcutaneous injection of 5 mg. of cortisone acetate (Cortone, Merck & Co. Inc., Rahway, N. J., U.S.A.) daily for 7 days. A dose of 1–2 mg. did not suffice for producing a satisfactory effect. Additional experiments were made on old female rats with the object of studying the influence of estradiol and progesterone injected in 1 mg. doses simultaneously with cortisone.

The measurement of oxidative phosphorylation was based on the general principles of the method of Maley and Lardy (Umbreit, Burris, Stauffer Manometric Techniques, 1957, p. 180). NaF was added to the reaction mixture to inhibit adenosine triphosphatase activity, and ADP was added to hasten the onset of the reaction. The reagents must be of the degree of purity required for analysis. The reagent solutions were prepared in twice-distilled water from which carbon dioxide had been removed by boiling. All the reagents were freshly prepared weekly. The substrates used were isocitric, α-ketoglutaric, succinic, malic and pyruvic acid. The amount of substrate added was 30μM. When α-ketoglutaric acid was used as substrate, 10μM of K malonate was added to prevent further oxidation of the succinic acid produced from α-ketoglutaric acid. The oxidation of pyruvic acid, again, was rendered more complete by the addition of fumaric acid (0.15μM of pyruvic acid +0.15μM of fumaric acid). All the preparatory work for measurement and the storage of the reagents took place in a refrigerated room. The following mixture was pipetted into cold Warburg flasks:

Reaction Mixture	*μM per 2.6 ml.*
0.6 ml 0.42 M sucrose	250.0
0.3 ml 0.1 M α-ketoglutaric acid	30.0
0.1 ml 0.1 M K malonate	10.0
0.1 ml 3×10^{-4} M cytochrome C	0.03
0.45 ml 0.05 m KH_2PO_4	22.5
0.05 ml 0.06 M ATP	3.0
0.1 ml 0.03 M ADP	3.0
0.1 ml 0.4 M NaF	40.0
0.1 ml 0.015 M $MgSO_4$ (added last)	1.5
0.5 ml mitochondria in 0.25 M sucrose	
Side-arm: 0.2 ml. of 0.25 M glucose with 4 mg. of hexokinase (Hexokinase Sigma, Type III, 150,000 units/gm.)	50.0
Center well: 0.2 ml. of 20% KOH and a small pice of filter paper.	

Immediately on addition of the mitochondria, the Warburg flasks were connected to the manometers and placed into a Warburg rack. The measurements were made in duplicate at 30° C. Temperature equilibration time was 7 minutes, after which the hexokinase-glucose in the side-arm was poured into the reaction mixture. The manometers were closed and the initial readings noted. At the same time the O bottles were removed from the Warburg rack for analysis of the inorganic phosphate. After incubation for 40 minutes the final manometer readings were noted and the proteins in the Warburg flasks were immediately precipitated by the addition of 0.4 ml. of 10% trichloroacetic acid, after which the flasks were transferred to the refrigerated room. Inorganic phosphate was determined from the cold trichloroacetic acid filtrate by the method of Fiske and Sub-

barow. The amount of inorganic phosphate utilized was calculated from the difference in the amounts of inorganic phosphate in the O test and in the corresponding experiment. The consumption of oxygen was calculated from the difference in the manometer readings.

The nitrogen was determined by the Kjeldahl method from the mitochondrial fraction used.

Treatment of Test Animals: The rats were anesthetized and bled by severing the renal vein. The liver was rinsed by injection of physiological saline solution, after which it was dissected out, weighed and placed on ice to cool.

All experimental animals have been fasted for 15 hours before they were killed.

Separation of Mitochondria: The cooled liver was homogenized rapidly in a homogenizer with an isotonic solution of sucrose (10× weight of liver). The use of sucrose solution as diluent prevents the aggregation of mitochondria and microsomes, separately or together, into larger particles. A lowering of the pH level during homogenization, which causes rapid enzymatic inactivation of the mitochondria, was prevented by buffering the sucrose solution with phosphate to pH 7.4, at which level the enzymes are more stable. Changes in the surface properties of the mitochondria, such as loss of selective permeability, which causes inactivation of the mitochondria, were prevented by the addition of ethylene diamine tetra-acetic acid, which chelates the calcium in the reaction mixture.

Fractionating centrifugation (Schneider) was used for separation of the mitochondria from the cells. The homogenate obtained was centrifuged in a cold centrifuge at 0° C, at first at a relatively low speed (700×g) for 10 min., during which the largest homogenate particles, cell debris and nuclei were sedimented. The supernatant was decanted and the residue was washed with buffered sucrose solution (5×weight of liver) and re-centrifuged at the same speed. The supernatant and the washings were added together. The resulting turbid solution was centrifuged at a higher speed (8,000×g), which caused precipitation of the mitochondria. After decanting, the solution was centifuged once more at the same speed. The combined sediments were suspended in buffered sucrose solution (7×weight of liver) and the mitochondria were homogenized by passing the solution twice through an injection needle. The suspension was centrifuged (12,000×g) for 10 min. and supernatant was decanted off together with any free flowing particulate material which was not firmly packed. The sediment was washed again in buffered sucrose solution, as above. The mitochondrial mixture to be used in the oxidative phosphorylation was prepared by suspending the purified mitochondrial sediment thus obtained in isotonic (8.5%) sucrose solution (1×original weight of liver).

Glucose-6-phosphatase and adenosinetriphosphatase activity of mitochondria suspension. A series of experiments were made to test the mitochondria suspension for glucose-6-phosphatase activity under the conditions of test, using the method of Swanson (4). Tests were also made for the presence of ATPase according to the method of Klein and Johnson (5). Neither of these enzymes showed any activity in the control rats or the cortisone-treated rats.

RESULTS AND DISCUSSION

The results of the experiments (284 determinations of oxidative phosphorylation on the control animals and 207 on the cortisone-treated animals) are shown in Figures 1–5 for each of the animal groups and the five substrates. Data are available on the consumption of oxygen (O/N) and phosphorus (P/N) per mg. of nitrogen and on their ratio (P/O), which, theoretically calculated, is 4 when α-ketoglutaric acid is used as substrate,

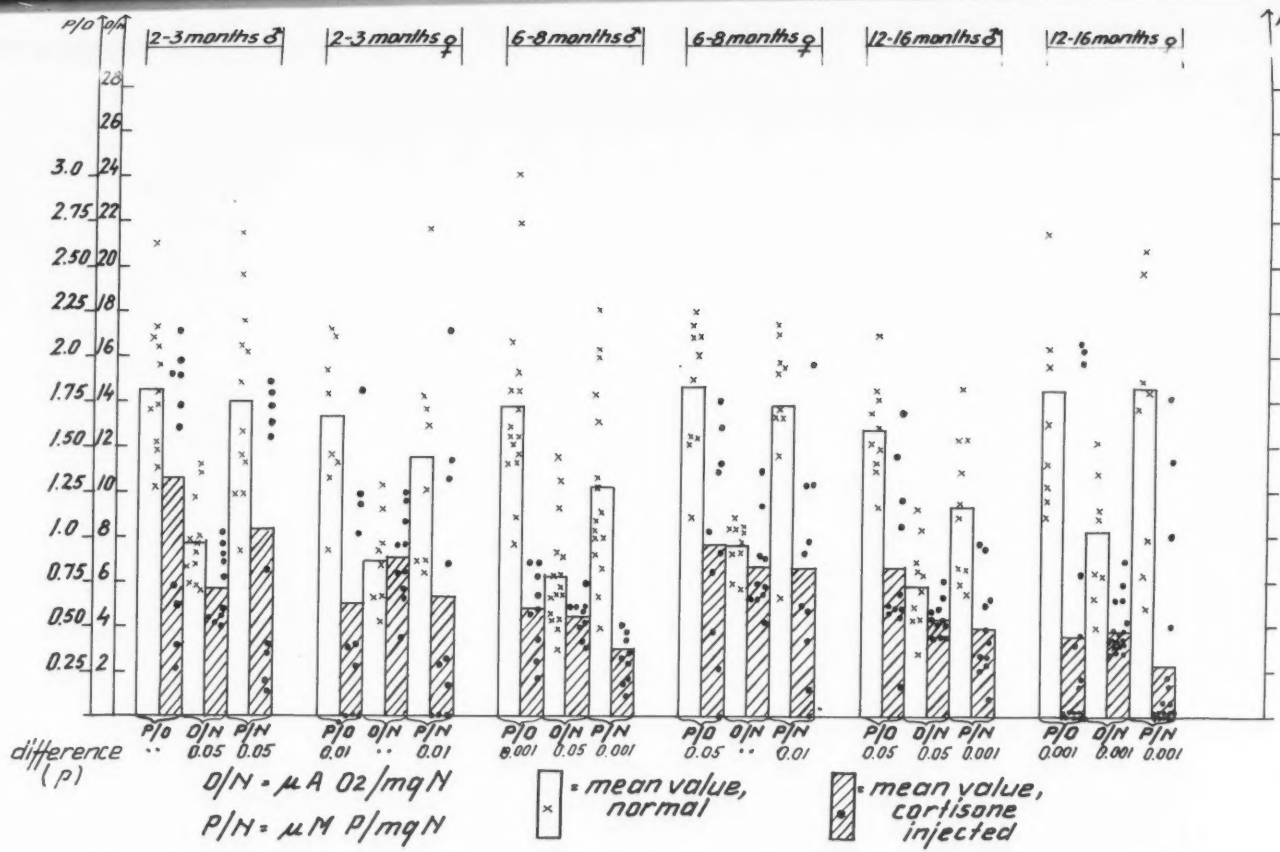

FIG. 1. Oxidative phosphorylation in rat liver mitochondria. Isocitric acid. The meaning of the probability calculus (p) under the figure: ·· = non significant. 0.05 = almost significant. 0.01 = significant. 0.001 = highly significant.

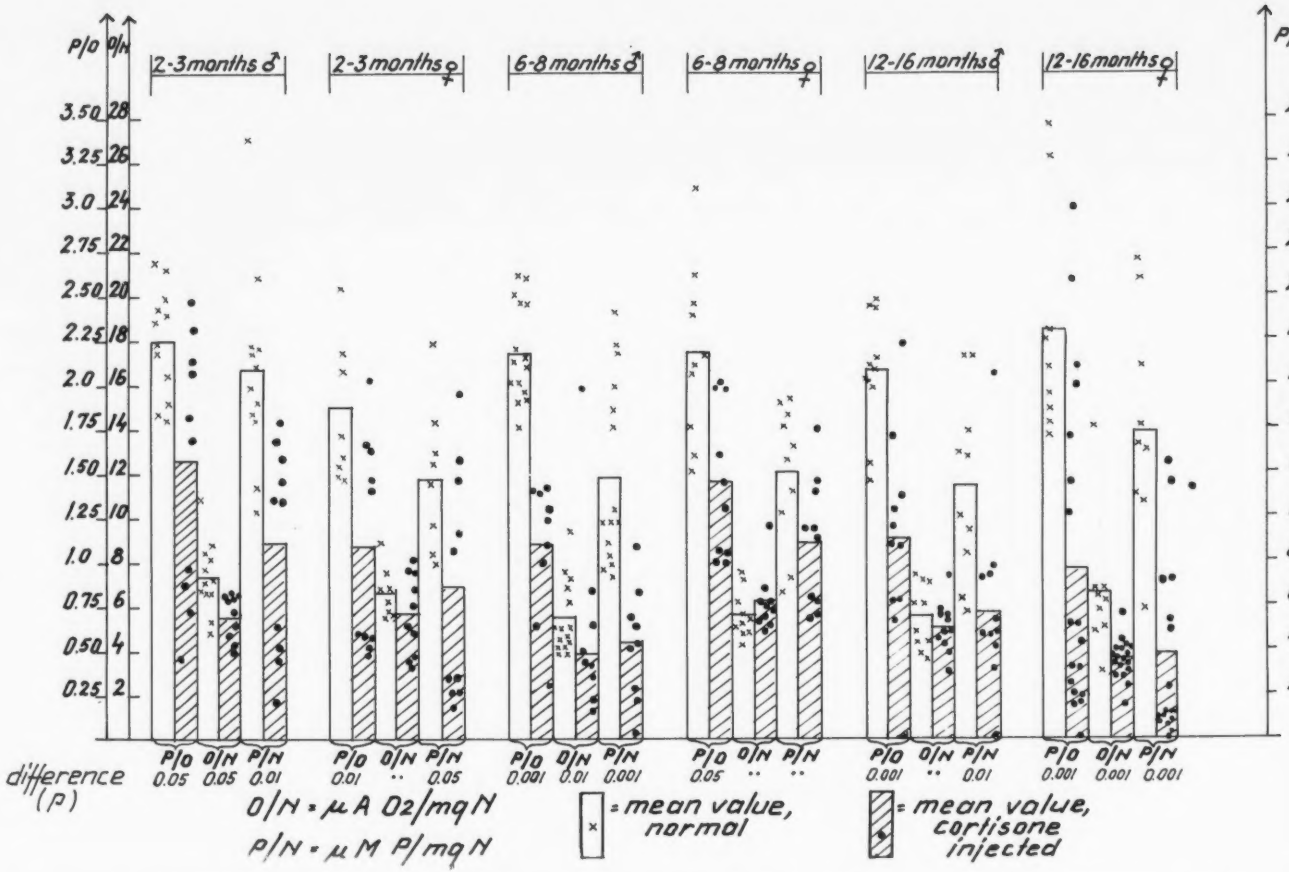

FIG. 2. Oxidative phosphorylation in rat liver mitochondria. α-Ketoglutaric acid. The meaning of the probability calculus (p) under the figure: .. = non significant, 0.05 = almost significant, 0.01 = significant, 0.001 = highly significant.

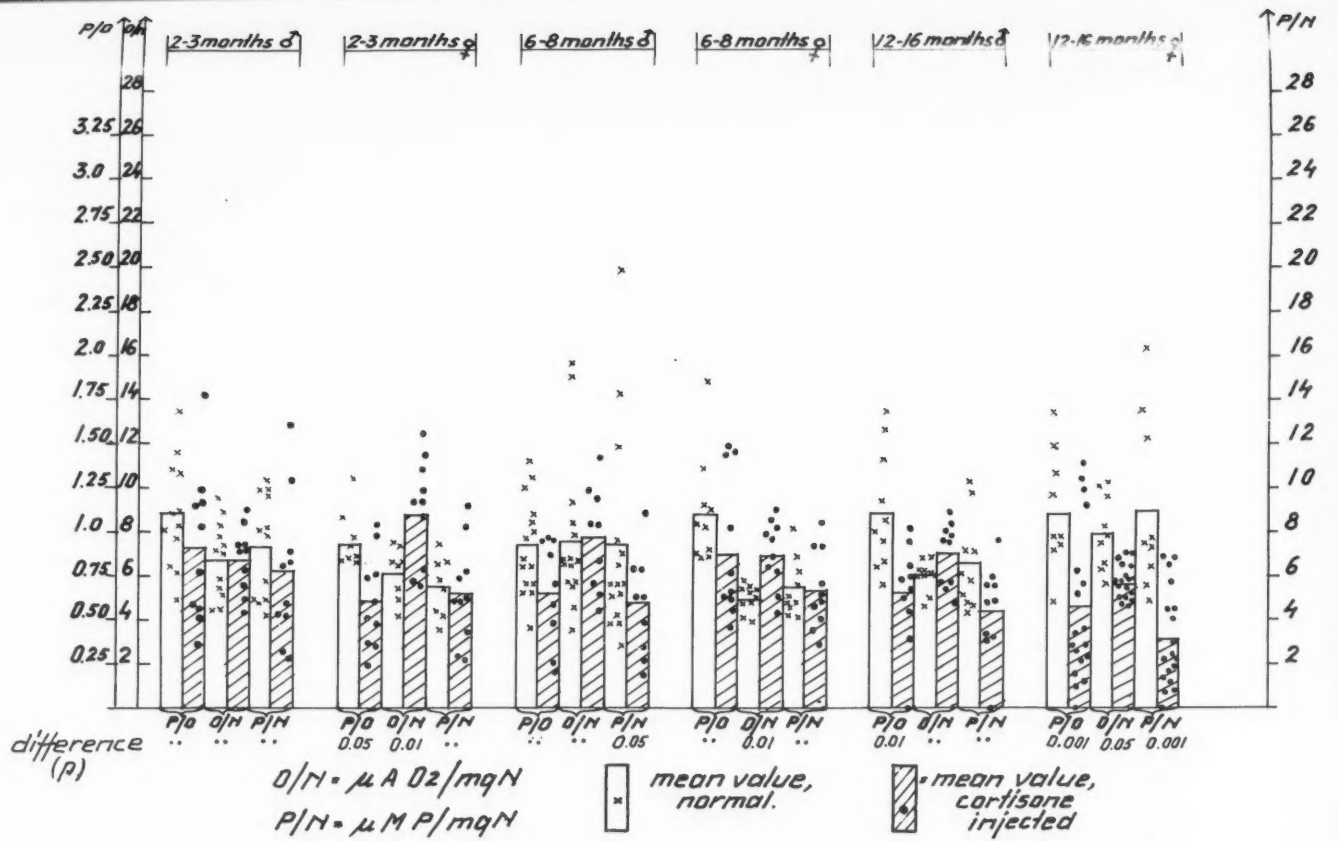

FIG. 3. Oxidative phosphorylation in rat liver mitochondria. Succinic acid. The meaning of the probability calculus (p) under the figure: ·· = non significant. 0.05 = almost significant. 0.01 = significant. 0.001 = highly significant.

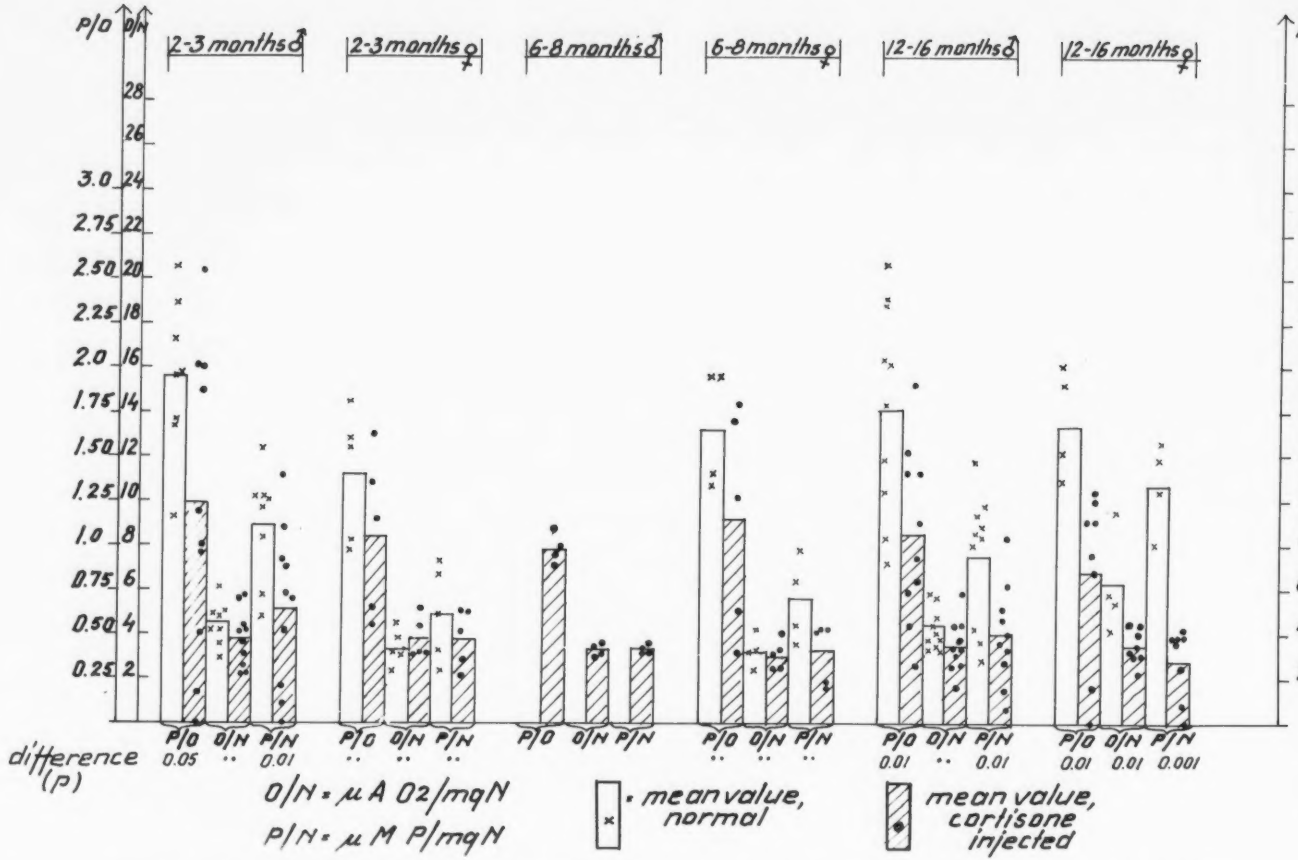

FIG. 4. Oxidative phosphorylation in rat liver mitochondria. Malic acid. The meaning of the probability calculus (p) under the figure: ·· = non significant, 0.05 = almost significant, 0.01 = significant, 0.001 = highly significant.

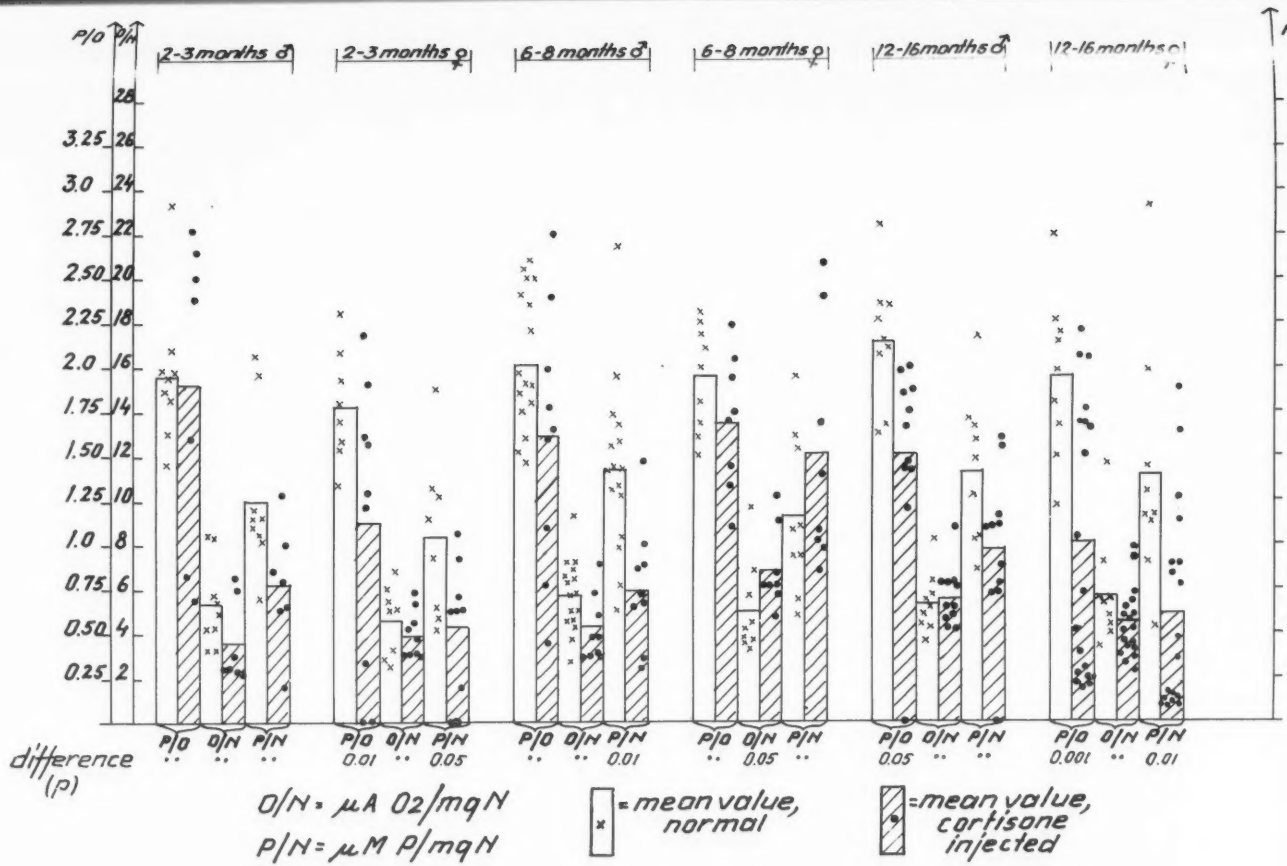

FIG. 5. Oxidative phosphorylation in rat liver mitochondria. Pyruvic acid. The meaining of the probability calculus (p) under the figure: $\cdot\cdot$ = non significant. 0.05 = almost significant. 0.01 = significant. 0.001 = highly significant.

3 for isocitric, malic and pyruvic acids, and 2 for succinic acid. The experiments were usually carried out on batches of five animals. The number of experiments in the different groups varied from 4 to 18.

The results in one and the same group sometimes varied greatly but often they were quite uniform. Differences occurred in both the control and the cortisone-treated animals. Thus it may be concluded that these variations in results are probably due chiefly to individual differences in mode of reaction.

Comparison of the mean values for oxidative phosphorylation in the cortisone-treated rats with the results for the controls shows that, when isocitrate was used as substrate, the oxygen consumption decreased to some extent ($P < 0.05$)[2] in all male animals. In female rats the same phenomenon was only elicited in old individuals, and in such cases it was highly significant ($P < 0.001$). Using α-ketoglutarate, the oxygen consumption was somewhat below normal in young male rats, significantly ($P < 0.01$) decreased in middle-aged males, and greatly decreased in old females. With succinate, the oxygen values were found to decrease slightly in old female rats and significantly in young and middle-aged females. When malate was used, there was a significant reduction in oxygen consumption in old female rats. Using pyruvate, the oxygen consumption was somewhat reduced in middle-aged female animals. As regards oxygen consumption in the different groups it may thus be said that a great decrease was obtained only in old female rats, and especially using isocitrate or α-ketoglutarate as substrate. Pyruvate usually caused no change in oxygen consumption.

The mean consumption of phosphorus, using isocitrate, was somewhat decreased in young male animals, significantly decreased in young and middle-aged females, and greatly decreased in middle-aged and old males and especially in old females. When the substrate was α-ketoglutarate, the phosphorus values were somewhat reduced in young female rats, significantly decreased in young and old male rats, and very greatly reduced in middle-aged male and old female rats. With succinate the phosphorus consumption declined somewhat in middle-aged male rats and greatly in old female rats. Using malate, the consumption of phosphorus decreased significantly in all the male animals and very greatly in old females. Using pyruvate, the values for phosphorus were somewhat reduced in young female rats and significantly reduced in middle-aged male and old female rat. Thus, in cortisone-treated animals, there was a significant decrease in the utilization of phosphorus, and in some of the experiments it was highly significant, when isocitrate or α-ketoglutarate was used as substrate. With succinate, malate and pyruvate the utilization of phosphorus in male ani-

[2] Deviations in both directions are in some instances included in the P-values of the mathematical analysis.

nals was variably inhibited. In the female groups the decrease was significant only in old animals, in which it was mostly highly significant.

The P/O ratio was nil in a total of 30 cases, 10 of which were female rats over 12 months of age, when isocitrate was used as substrate. The other O-values are distributed over the different groups. In the control animals, however, no O-values were obtained. The P/O ratio was nil in all the cases: no phosphorus consumption could be demonstrated, whereas oxygen was always consumed. If the consumption of oxygen declines at the same time as that of phosphorus, it tends to raise the P/O ratio. When isocitrate was used as substrate, the ratio was somewhat reduced in old male and middle-aged female rats as compared with the mean ratio for the control animals. In young females the decrease was significant and in middle-aged male and old female rats highly significant. Using α-ketoglutarate, the decrease in P/O ratio was slight in young male rats, significant in young and middle-aged female rats, and highly significant in middle-aged male and in old animals. In the case of succinate the decrease in the ratio was slight in young females, significant in old males, and great in old female rats. With malate some decrease was obtained in young males and a significant decrease in old animals. With pyruvate the ratio was somewhat low in old male rats, significantly decreased in young females, and greatly decreased in old female rats. Comparison of the P/O ratios in the various groups thus showed that the ratio was reduced in young female and old male rats and particularly in old female rats.

The liver of the rats treated with cortisone was examined in all cases with respect to size, colour, consistency, and fat content. There were well-marked changes in the liver in 30 cortisone-treated rats. The colour had become much paler, consistency was fragile, and the cut surface granular and fatty. Most of these cases were in the group of old female rats. The phenomenon also occurred in some of the young female rats. In the group of male rats the changes were more rare and were evenly distributed with regard to age. A study of the values for oxidative phosphorylation obtained in the animals concerned shows that the uncoupling effect of cortisone on oxidative phosphorylation was highly significant in most cases; in the rest—a small number—it was significant.

The above findings permit the conclusion that cortisone very frequently inhibits oxidative phosphorylation in liver mitochondria of rats of all ages and both sexes. Female rats over 12 months old showed a definitely stronger effect than the other groups: this was the only group in which consumption of oxygen was also significantly reduced. The highest degree of uncoupling of oxidative phosphorylation was obtained when isocitrate or α-ketoglutarate was used as substrate. In old female rats the uncoupling was highly significant with all the subtrates used. The P/O ratio was also definitely lower in old female rats than in the other groups.

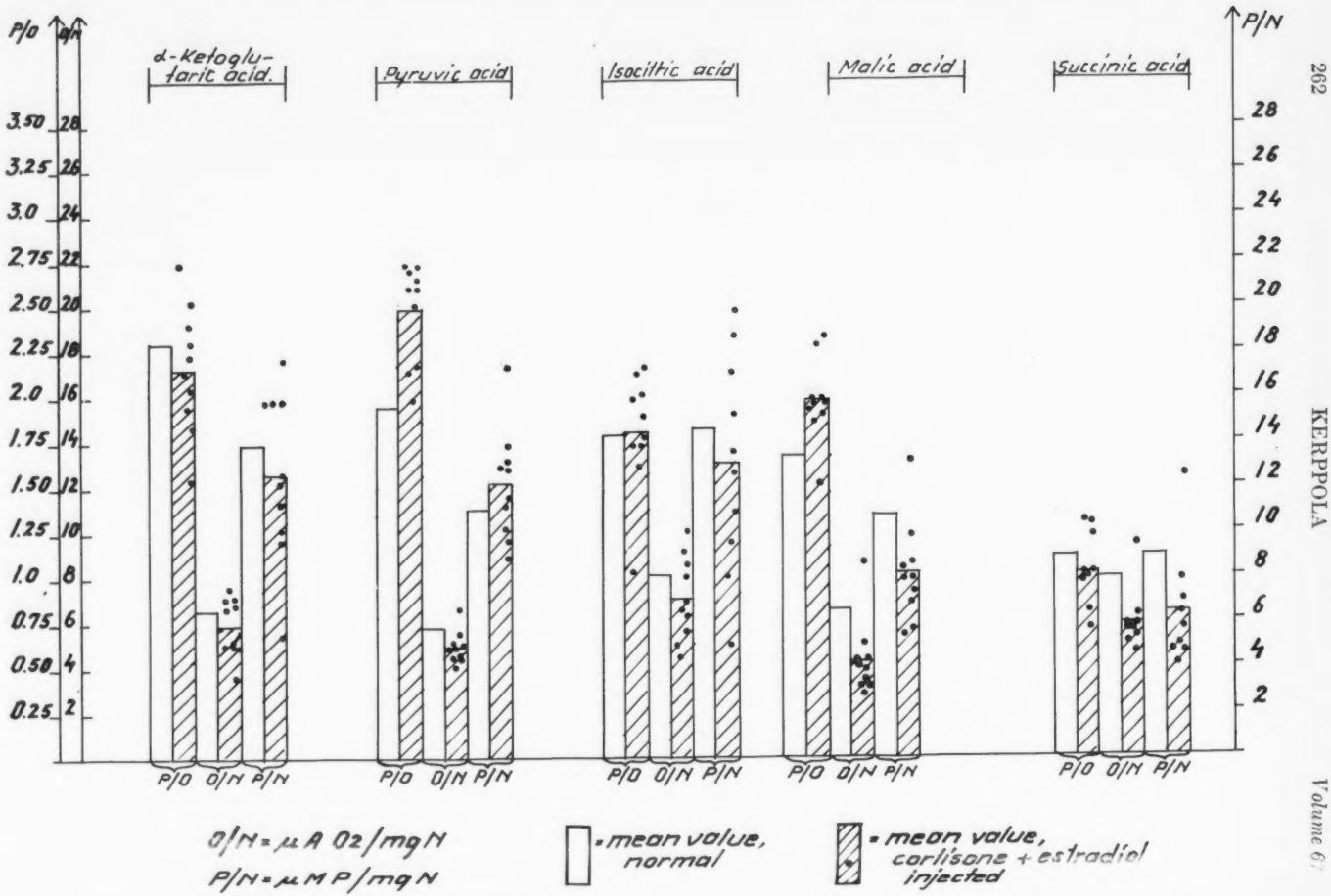

Fig. 6. Oxidative phosphorylation in rat liver. Mitochondria incubated 15 minutes

Study of the effect of female hormones, injected simultaneously with the cortisone into old female rats, showed that progesterone (5 cases) raised the phosphorus values and the P/O ratio slightly. Estradiol (10 cases) markedly counteracted the uncoupling effect of cortisone on oxidative phosphorylation with all substrates used (Fig. 6). This finding is in agreement with the above statement that the uncoupling effect of cortisone is relatively weak in middle-aged female rats of reproductive age, and strong in old female rats. The normal oxidative phosphorylation seen in some experiments on 12-month-old female rats despite the cortisone treatment may, in the light of the above results, be due to persisting ovarian function in these individuals.

Growth hormone (Somatropin) and androgenic hormone (19-nor-androstenolon phenylpropionate) exerted no effect on middle-aged male rats when injected simultaneously with cortisone. Similarly, testosterone was ineffective in old female rats.

A large proportion of tissue ATP—the most important source of the dynamic energy in all vital processes—is produced through oxidative phosphorylation. When cortisone retards oxidative phosphorylation, the production of ATP is inhibited correspondingly. This may result, among others, in impairment of hexokinase function and a disturbed sugar metabolism similar to diabetes. A decreased phosphorylase activity due to lack of ATP may impair the mobilization of glycogen from the tissues. For the same reason, the oxidation of fatty acids in the Krebs cycle, as well as protein synthesis, may be inhibited. It thus seems that the uncoupling effect of cortisone on oxidative phosphorylation may account for many of the clinical symptoms seen in cases of hypersecretion or excess dosage of cortisone.

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NOTES AND COMMENTS

THE BURSA OF FABRICIUS AS AN INDICATOR OF CHRONIC STRESS IN IMMATURE CHICKENS*

ABSTRACT

Repeated injections of ACTH or repeated stress in the form of immobilization resulted in a statistically significant regression in weight of the bursa of Fabricius in young chickens. Repeated injections of plasma from immobilized birds into other birds resulted in a similar regression when compared with bursae from birds injected with plasma from non-stressed birds. Results indicate that bursal regression can be used as an indicator of chronic stress in young chickens.

INTRODUCTION

There are ample data in the literature to indicate that the criteria of adrenal cortex mobilization generally utilized in response to injections of ACTH or application of stressors in mammals are not entirely applicable to birds (1–11). Two physiologic indicators of an *acute* response to injection of ACTH and/or to stressors in birds have been described: (a) acidophilia in both young (12, 13) and mature (14) chickens and (b) adrenal ascorbic acid depletion in adult birds (15) and in bursectomized young birds (16). In contrast to the availability of these criteria of acute (within 24 hours) mobilization of adrenal cortical tissue in the bird, no satisfactory indicators are available for evaluating the effects of repeated injections of ACTH or of chronic stressors; the purpose of the present experiments is to demonstrate the utility of the bursa of Fabricius as an indicator of chronic stress.

MATERIALS AND METHODS

Young White Leghorn cockerels housed in battery brooders and fed and watered *ad lib.* were used in all experiments.

ACTH (Armour's *Adrenomone*) diluted appropriately with 16% gelatin was the hormone source for Groups 1, 2 and 3 wherein the experimental birds were injected intramuscularly daily with various doses. In Group 1, injected control birds received only 16% gelatin.

Restraint in Groups 4 and 5 consisted of fastening birds to boards on their backs with rubber bands for one or six hours. In Group 5, the experimental birds were injected intracardially with 2 ml. heparinized plasma from other birds which had been immobilized for one hour immediately preceding withdrawal of blood; control animals were injected with 2 ml. plasma from similar birds not immobilized immediately preceding withdrawal of blood. Each bird in Group 5 received blood from a different donor (which was not used as a donor again, i.e. each donor bird was used only once).

At autopsy, the bursae were removed and weighed immediately on a Roller-Smith torsion balance.

RESULTS AND DISCUSSION

Results of injecting 0.05, 5 or 6 I.U. ACTH/day for 10 or 12 days (Table 1) indicate that the bursa of Fabricius in young cockerels decreased in size as compared to either injected or non-injected control birds of similar age. The magnitude of decrease was from 22% (Group 2) to 66% (Group 3). Since it has been shown previously (17, 18) that repeated injections of various adrenal steroids (as well as progesterone and testosterone) resulted in decreased weights of bursae, the decrease in bursal weights obtained here can be assumed to be a result of the stimulating effect of exogenous ACTH on adrenal cortical tissue in causing an increased secretion of steroids by the birds' adrenals (19).

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TABLE 1. EFFECT OF VARIOUS TREATMENTS ON ADRENAL AND BURSAL WEIGHTS

	Group and treatment	Duration (days)	Mean body weight (gm.)	Number birds	Adrenal weight[1] (mg.%)	Bursa	
						Weight[1] (mg.%)	Decrease (%)
1	Injected controls		88	9	18.8 ± 3.7	291 ± 74	
	0.05 I.U. ACTH/day	10	77	8	22.2 ± 3.1	218 ± 62*	25
	5 I.U. ACTH/day	10	81	10	20.8 ± 6.3	221 ± 75*	24
2	Uninjected controls		246	10	18.1 ± 3.5	484 ± 108	
	0.05 I.U. ACTH/day	10	218	10	20.4 ± 3.4	376 ± 119***	22
	5 I.U. ACTH/day	10	206	10	21.5 ± 3.6	278 ± 75***	43
3	Uninjected controls		224	10	17.1 ± 2.8	565 ± 97	
	6 I.U. ACTH/day	12	168	10	23.1 ± 4.3***	188 ± 84***	66
4	Controls		267	10	15.4 ± 1.8	511 ± 135	
	Restraint 6 hrs./day	10	238	10	17.9 ± 3.4	349 ± 101**	32
5	Controls 2 ml. plasma from unstressed birds	10	363	10	17.3 ± 18.0	456 ± 101	
	Normals 2 ml. plasma from restrained (1 hr.) birds	10	396	9	17.9 ± 3.5	338 ± 72**	26

[1] Mean ± standard deviation.
* $P < .05$.
** $P < .01$.
*** $P < .005$.

The data in Table 1 also show that repeated restraint (immobilization) for six hours/day for 10 days (Group 4) resulted in a decrease of bursal weights comparable to the decrease obtained with injection of ACTH (Groups 1, 2 and 3). Restraint presumably resulted in mobilization of adrenal cortical tissue via ACTH (13). That the decrease in weights of bursae was the result of some blood-borne material is evidenced by securing blood plasma from birds that had been restrained for one hour and immediately injecting it intracardially into recipient birds. Birds so treated for 10 days had smaller bursae than control birds treated similarly with plasma from unrestrained donor birds. The identity of the agent present in the plasma of restrained birds but not in plasma of unrestrained birds responsible for the decrease in weight of bursae is not known. Possibilities are any or combinations of the following: ACTH, adrenal cortical steroids or other materials such as histamine or epinephrine which have been shown to stimulate the adrenal cortical tissue directly or indirectly (13).

The nature of the bursal regression appears to be grossly similar to thymic (or other lymphatic tissue) involution which is characteristic of adrenal steroid action in mammals. Whether the bursa of Fabricius is typical lymphatic tissue has not been determined; there is evidence pro (18, 20) and con (21).

Although the mean adrenal weight of each treated group was larger than that of the respective controls, the difference was statistically significant only for the birds of Group 3. These data substantiate the relative insensitivity of adrenal weight as an indicator of stress in birds.

The above data indicate that the bursa of Fabricius can be utilized as a criterion of adrenal cortical stimulation by repeated injections of ACTH or repeated (or continuous) application of stressors in young chickens. The use of this criterion is limited, however, by the fact that the bursa atrophies as maturity progresses.

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LOCALIZATION OF THE RENAL TUBULAR ACTION OF PARATHYROID HORMONE[1]

ABSTRACT

Administration of a purified parathyroid extract to the parathyroidectomized dog resulted in a marked increase in the urinary excretion of phosphate accompanied by a significant fall in its tubular reabsorption, in the absence of any increase in the filtered load of phosphate. Employing the stop-flow technique, it was demonstrated that parathyroid extract increased phosphate excretion probably by affecting the reabsorption of phosphate in both proximal and distal segments of the nephron.

The phosphaturic effect of parathyroid extract has been demonstrated repeatedly since 1925 (1, 2, 3, 4). There exists, however, no general agreement on the mode and the site of action of the extract. In 1929, Albright and Ellsworth (2) suggested that parathyroid hormone increases the urinary excretion of inorganic phosphate by a direct action on the renal tubules, a view supported by recent work of Beutner and Munson (5) showing the great rapidity and magnitude of the effect in parathyroidectomized rats. Some other investigators (6, 7, 8, 9), however, have maintained that the phosphaturic effect of parathyroid extract should be attributed to alterations in renal hemodynamics resulting from the presence of impurities in the crude extract.

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The current availability of more highly purified parathyroid hormone preparations has stimulated the re-examination of possible direct renal effects. Lavender and his associates (10) have recently shown that the injection of small amounts of purified parathyroid hormone into one renal artery of dogs was followed by a significant increase in the urinary excretion of phosphate from the treated kidney alone.

The present investigation was initiated to study the possible renal action of a purified parathyroid extract in parathyroidectomized dogs and to localize the tubular sites at which it may exert its effect.

METHODS

Female mongrel dogs weighing between 14–18 kg. were anesthetized with pentobarbital and a total thyroparathyroidectomy was performed. Twenty to 36 hours later, the stop-flow technique of Malvin *et al.* (11) was employed for the localization of tubular action of the extract. Through a right flank incision a ureteral catheter was tied in place. When a brisk mannitol diuresis of about 10 ml./minute per kidney was achieved, three clearance periods of three minutes each were carried out. The catheter was then clamped for four minutes and following release of the clamp serial samples of small volumes of urine were collected over the subsequent three minutes. The experiment was concluded with three final clearance periods. Two stop-flow procedures were performed in succession before and twenty minutes after the injection of 200–400 units of parathyroid extract (12, 13). Preliminary experiments had shown that the phosphaturic effect of the extract could be best demonstrated with an endogenous phosphate load. We also found that the presence of mannitol interfered with the determination of the very small amounts of phosphate present in some of the stop-flow samples. Hence P^{32} was used to facilitate the determination of small quantities of phosphate. Ten minutes before starting the experiment the animal was given a priming dose of 150 μcuries of P^{32} and a sustaining infusion containing 2.5 μcuries of P^{32} per minute. The amount of phosphate in the stop-flow samples was calculated from specific activities obtained during the free-flow periods.

RESULTS

As shown in Table 1, the administration of the extract resulted in a substantial fall in the rate of glomerular filtration (GFR) and urine flow. (All data in the table are given for one kidney.) Despite a decrease in GFR, plasma concentration of phosphate, and urine flow, the excretion of phosphate increased from 0.26 mg./minute to 0.36 mg./minute. The reabsorption decreased accordingly from 1.04 mg./minute to 0.06 mg./minute. When the reabsorption of phosphate was expressed in terms of the filtered load, it decreased from a control value of 82% to 14% following injection of the parathyroid extract.

TABLE 1. THE EFFECT OF PARATHYROID EXTRACT ON RENAL EXCRETION OF PHOSPHATE

V, ml./min.	GFR, ml./min.	Plasma PO_4, mg.%P	Urine PO_4, mg.%P	Filt. load, mg./min.	Phosphate as P		
					Excretion, mg./min.	Reab., mg./min.	% Filt. reab.
Infusion: 20% mannitol, 0.85% NaCl at 10 ml./min.; creatinine 0.5% at 5 ml./min.							
10.3	26.2	4.8	2.12	1.26	0.22	1.04	82
11.0	26.5	4.7	2.10	1.26	0.23	1.03	82
11.0	25.4	4.7	2.10	1.19	0.23	0.96	81
10.0	23.4	4.0	1.80	0.92	0.18	0.74	80
10.3	23.1	4.0	1.80	0.92	0.19	0.73	80
10.3	23.5	4.1	1.76	0.96	0.18	0.78	81
Infusion: As above; 400 units PTE							
7.6	16.5	3.8	3.00	0.63	0.23	0.40	63
7.6	16.0	3.4	3.40	0.55	0.26	0.29	53
7.3	14.8	3.1	3.70	0.46	0.27	0.19	41
6.3	14.2	2.5	4.80	0.36	0.30	0.06	17
6.3	14.1	2.8	5.08	0.39	0.32	0.07	18
6.1	13.5	3.1	6.00	0.42	0.36	0.06	14

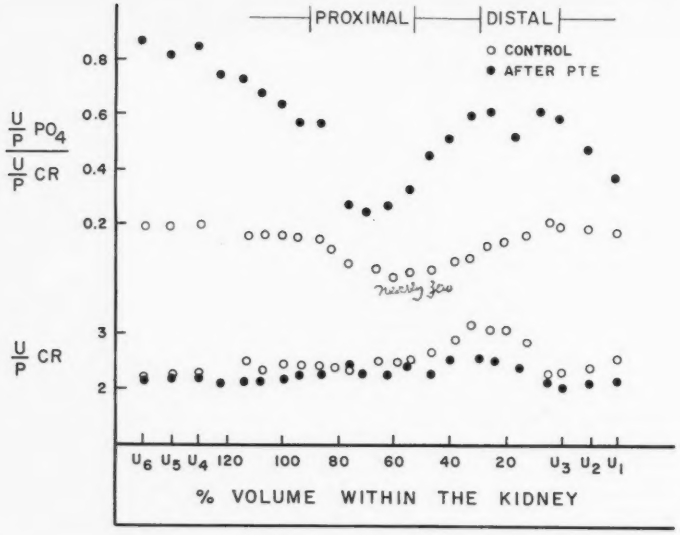

FIG. 1. Comparison of phosphate reabsorption in a stop-flow experiment in the parathyroidectomized dog in the control state and twenty minutes following the administration of 400 units of purified parathyroid extract.

Figure 1 depicts the results of a stop-flow experiment in the same parathyroidectomized dog. On the ordinate is plotted the urine to plasma (U/P) concentration ratio for creatinine and the U/P concentration ratio for phosphate (U/P_{PO_4}) divided by the simultaneously determined U/P ratio for creatinine (U/P_{Cr}). A value less than one for this ratio implies tubular reabsorption and a value greater than one implies net tubular secretion. On the abscissa is plotted the per cent volume of fluid trapped in the kidney during the stop-flow period (14).

Comparison of the excretion pattern of phosphate before and after the administration of parathyroid extract shows the following significant features. Phosphate was reabsorbed most avidly in the proximal segments of the nephron of the parathyroidectomized dog before and after administration of the extract. The maximal value for $U/P_{PO_4}/U/P_{Cr}$ was 0.203 in the distal samples and fell practically to zero in the proximal samples, thus indicating an almost complete reabsorption of phosphate in the proximal segments of the nephron of the parathyroidectomized dog. Twenty minutes after administration of the extract, $U/P_{PO_4}/U/P_{Cr}$ increased to 0.614 in the distal and 0.735 in the proximal samples. The lowest value for $U/P_{PO_4}/U/P_{Cr}$ observed following the injection of the extract was 0.253, somewhat higher than the maximum ratio obtained during the control stop-flow experiment. It is also worthy of note that in both stop-flow samples we did not observe any $U/P_{PO_4}/U/P_{Cr}$ which was greater than one.

DISCUSSION

The results of our experiment confirm the findings of Lavender (10) by demonstrating the marked phosphaturic action of a highly purified parathyroid extract. The increase in excretion of phosphate in our experiment was more impressive than that noted by Lavender, probably because the present investigation was carried out in the parathyroidectomized dog. The increase in phosphate excretion occurred within 10 minutes

in this dog and appeared to continue even after 30 minutes following administration of the extract. The increase in phosphate excretion is clearly due to a marked decrease in the tubular reabsorption of phosphate and not due to any increase in the filtered load of phosphate. In fact, the administration of the extract decreased both the rate of glomerular filtration and the plasma concentration of phosphate.

It is evident from Table 1 that only 14% of the filtered load of phosphate was being reabsorbed by the renal tubules following administration of the extract. This finding *per se* suggests that, regardless of any distal tubular effect, the parathyroid extract must also exert some effect on the proximal tubules, assuming that there is no net tubular secretion of phosphate. We feel that this assumption is justified in view of the stop-flow experiments with phosphate (11, 14). Our stop-flow data, as shown in Figure 1, seem to offer further evidence in support of this contention by showing that injection of the parathyroid extract increased $U/P_{PO_4}/U/P_{Cr}$ in both proximal and distal segments of the nephron. These observations indicate that parathyroid hormone probably affects the reabsorption of phosphate in both segments of the nephron. It must be remembered, however, that the tubular fluid from the proximal segments must pass and probably be modified by the distal tubules. But the increase in value of $U/P_{PO_4}/U/P_{Cr}$ in the proximal samples was of such magnitude that it probably represented an actual decrease in the reabsorption of phosphate in the proximal segments of the nephron. In contrast to the findings of Nicholson (15) we were unable to find any evidence of tubular secretion of phosphate during the clearance or the stop-flow periods of the parathyroidectomized dog before or after the injection of the extract.

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INITIATION OF LACTATION IN RABBITS FOLLOWING PLACEMENT OF HYPOTHALAMIC LESIONS[1]

ABSTRACT

Destruction of the medial basal tuberal region of the hypothalamus of ovariectomized female New Zealand White rabbits whose mammary glands had been suitably developed led to initiation of milk secretion. Since this same hypothalamic area controls the release of pituitary ovulating hormone, it is suggested that the neural or neurohumoral factor(s) stimulating the discharge of gonadotropin inhibit(s) lactation by blocking the release of lactogenic hormone. Once this inhibition is removed lactogenesis occurs.

In most mammals the initiation of milk secretion is dependent upon the combined effects of the ovarian steroids and pituitary growth hormone, ACTH, and most important, lactogenic hormone (Prolactin, luteotrophin, LTH) (1). The liberation of lactogenic hormone appears to be under chronic inhibition, directly or indirectly, by the central nervous system. Removal of the pituitary gland from central nervous (hypothalamic) influence can be achieved by transplanting it, *e.g.* to the kidney capsule. In the rat such transplantation results in continuous activity of the corpora lutea by fostering secretion of luteotrophin (2). In the rabbit drugs like reserpine activate lactation (3, 4, 5) apparently by antagonizing the hypothalamic inhibition and permitting an enhanced secretion of lactogenic hormone. The present experiments attempt to emulate the reserpine effect by means of localized electrolytic lesions in the hypothalamus.

METHODS

Large ovariectomized New Zealand White rabbits were employed in this study. The effect of estrogen (estradiol benzoate, 0.1 mg., s.c., daily for ten days, or every other day for three weeks) on mammary development and milk secretion was evaluated by visualizing the glands through a mid-ventral skin incision and rating them according to the method of Gardner and Turner (6). Electrolytic lesions were then placed in the hypothalamus by stereotaxic methods (7). In the acute experiments (10 days of estrogen priming) the lesions were made the day following the mammary gland inspection. Three days after lesioning, the glands were again inspected and compared with their earlier condition. In chronic experiments (estrogen for three weeks) the lesions were placed a week after the first mammary gland evaluation. After a minimum of two more weeks for recovery from the traumatic effects of the lesions, estrogen treatment was repeated and its influence on the glands was compared with that prior to the lesions.

RESULTS

Reconstruction of the acute lesion sites from histological preparations of the brains showed that maximal mammary activation—as evidenced by presence of milk in the ducts and lobule-alveolar systems—occurred in those animals in which the lesions destroyed the medial basal tuberal region. Little or no milk production resulted from lesions placed elsewhere, as shown in the bar graphs of Figure 1. In only 6 of 24 "chronic" rabbits was lactation initiated, and their lesions were again all restricted to the medial basal tuberal region. The lesions of the 18 "chronics" which failed to lactate were all found to lie outside the "effective" area.

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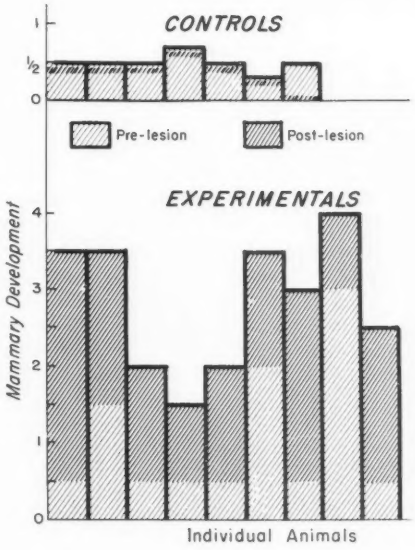

FIG. 1. Mammary development and milk production (graded on a scale of 0 to 4+) before and after hypothalamic lesions. (Results of acute experiments only.) *Controls* (7 rabbits): lesion at site other than medial basal tuberal region. Note regression in one case. *Experimentals* (9 rabbits): lesion in medial basal tuberal region.

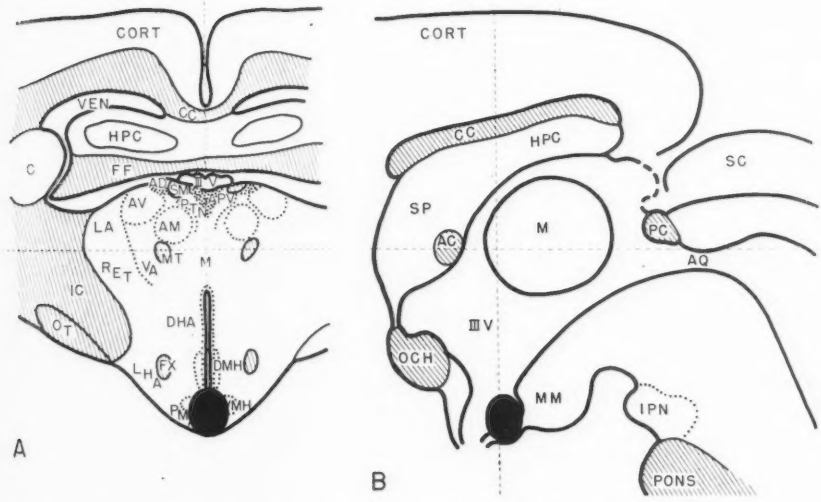

FIG. 2. Reconstruction of the rabbit diencephalon in (A) cross-section and (B) midsagittal projection, indicating the site of lesions which resulted in mammary activation. DMH—N. dorsomedialis hypothalami; FX—Fornix; LHA—Lateral hypothalamic area; (V)MH—N. ventromedialis hypothalami; MM—N. mammallaris medialis; OCH—Optic chiasma; PM(A)—Premammillary area.

Figure 2 shows the area common to all lesions which were followed by activation of the mammary glands. The crucial site is indicated by the blackened oval in this diagrammatic cross-section and sagittal reconstruction of the diencephalon. The "effective" lesions destroyed the midline nuclei of the posterior basal tuberal region, namely N. periventricularis arcuatus and the base of N. ventromedialis hypothalami (as well as, in some instances, the posterior part of the median eminence).

DISCUSSION

The results may be interpreted as confirmation of the hypothesis that the discharge of lactogenic hormone is held in check by inhibitory nervous influences. The inhibitory mechanism would appear to reside in or to project through the basal tuberal hypothalamus. The fact that this very same hypothalamic site controls the discharge of pituitary ovulating hormone (presumably largely luteinizing hormone, LH) lends support to the suggestion of Everett (9) that the same humoral substance(s) traversing the hypophysial portal system to stimulate LH discharge inhibit(s) release of lactogenic hormone. Physiological release of lactogenic hormone (*i.e.* normal post-partum lactogenesis) would imply a depression of this part of the brain. A peculiar type of electroencephalographic record related to behavioral depression has been reported in the female rabbit brain by Kawakami and Sawyer (10) following treatment with oxytocin in dosages observed by Haun (11) to result in lactogenesis. Whether afferent stimuli (*e.g.*, those related to parturition or suckling) promote lactogenesis by secondary inhibition of the brain following the reflex discharge of oxytocin is a question for further study.

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INHIBITION OF THYROTROPIC ACTIVITY BY IMMUNOLOGICALLY SPECIFIC ANTISERUM[1]

ABSTRACT

The preparation of rabbit antisera against bovine thyrotropin is described. These antisera are immunologically specific and inhibit the increased thyroidal uptake of I^{131} in mice treated with bovine thyrotropin.

The availability of relatively pure preparations of anterior pituitary hormones has permitted the successful production of antisera with anti-hormonal activity. Specific antibodies to bovine somatotropin (STH) and to porcine adrenocorticotropin (ACTH) have been reported (1, 2). Antisera to other pituitary hormones have been prepared which react with the hormone used as antigen but lack serologic specificity (3). In the present study, potent antisera were prepared to each of two bovine thyrotropin (TSH) preparations. These antisera were immunologically specific and inhibited thyrotropic activity when assayed in mice.

MATERIALS AND METHODS

Serology

Female rabbits were immunized intradermally with complete Freund's adjuvant containing 1–2 units of bovine thyrotropin ("Thytropar" or U.S.P. Thyrotropin Reference Standard). Four weeks later, an intraperitoneal booster dose of 2–3 units[2] of TSH was given, and after a further 2 weeks the animals were bled. The immunologic specificity of the antisera was determined by a modification of Stavitsky's tanned erythrocyte hemagglutination assay (4), by complement-fixation and by the Ouchterlony gel diffusion technique (5). The sera were tested against the TSH preparations used for immunization; bovine ACTH, STH and posterior pituitary extract; ovine lactogenic (LH) and follicle stimulating hormone (FSH[3]; and human ACTH[4]. The erythrocytes in the hemagglutination test were sensitized with solutions of 0.1 or 1.0 unit/ml. of TSH. The concentrations of other hormones used for sensitization varied between 0.1 and 1.0 unit/ml. for bovine ACTH and 0.1 and 1.0 mg./ml. for the other pituitary hormones.

Hemagglutination-inhibition assays were performed by incubating aliquots of rabbit antiserum with serial dilutions of TSH in saline or in human albumin solution (50 mg./ml.). Incubation was at 37° C for 30 minutes and then at 4° C for 4 hours. The mixtures were then serially diluted and used as the antisera in the standard hemagglutination test.

Anti-Hormonal Activity (Bio-Assay)

The anti-hormonal activity of the antisera was assayed by a modification of the method of Querido, Kassenaar and Lameyer (6) utilizing the thyroidal uptake of radioactive iodine (I^{131}) in the mouse. Mice weighing 15 to 20 gm. each were injected with 80 micrograms of *l*-thyroxine to suppress endogenous TSH secretion. They were then given 0.5 ml. of rabbit antiserum 1–4 hours before the administration of the first of 4 doses of bovine TSH totaling 24 milliunits over 48 hours. Immediately following the fourth injection of TSH, a tracer dose of approximately 1.0 microcurie of I^{131} was administered; 24 hours subsequently the mice were sacrificed, the thyroid glands removed and dissolved in 10% NaOH, and the I^{131} counted in a well-type scintillation counter. Controls with saline or normal rabbit serum were done at the same time. All injections were intraperitoneal.

Experiments were also carried out in which the supernatant fluid from a mixture of 0.5 ml. antiserum and 24 milliunits of TSH, which had been incubated for one hour at 37° C and

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[2] The term *units* used in this report refers to U.S.P. Thyrotropin Reference Standard Units.

[3] The bovine growth hormone and the ovine luteinizing hormone and follicle stimulating hormone were supplied by the Endocrinology Study Section, National Institutes of Health, under the Pituitary Hormone Distribution Program.

[4] Kindly supplied by Dr. Maurice Raben, Tufts University School of Medicine, Boston, Massachusetts.

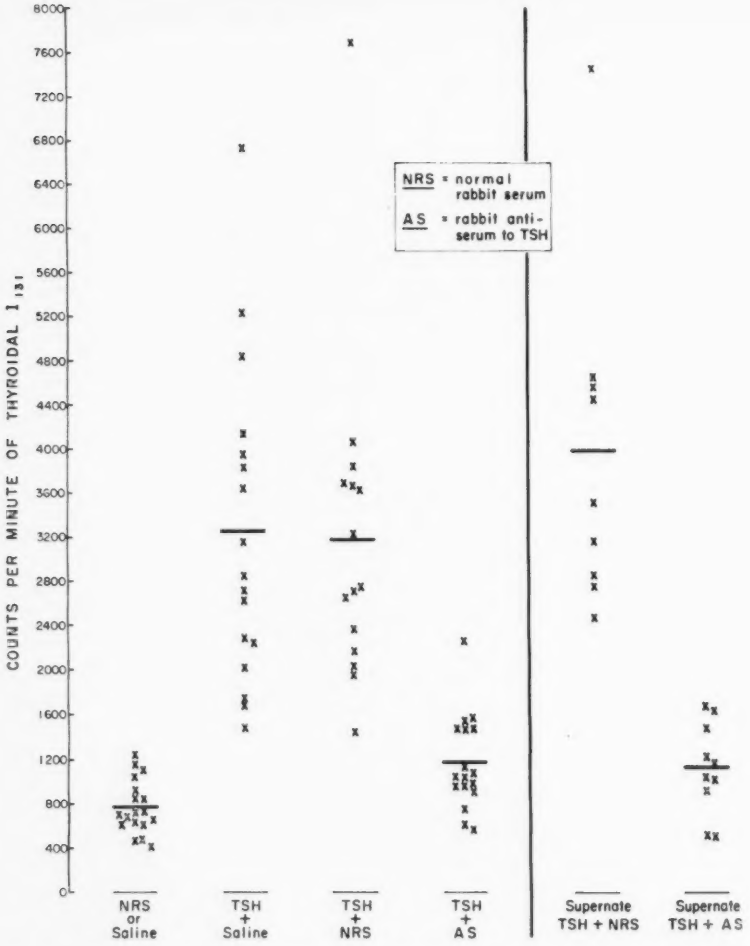

FIG. 1. The inhibitory effects of rabbit antiserum to bovine thyrotropin administered prior to TSH stimulation (columns to left of vertical divider) or after removal of TSH by precipitation (columns to right of divider). Each "x" represents a single mouse. The horizontal bars in each column indicate the mean values for that group.

then left overnight in the refrigerator, was injected into each mouse. This was given in 4 intraperitoneal injections over 48 hours and the 24-hour uptake of I^{131} by the thyroid was then determined as described. A control group received an incubated mixture of normal rabbit serum and TSH.

RESULTS

When tested by the hemagglutination assay, the antiserum to "Thytropar" had a titer of 1:800 with this hormone as antigen. Antiserum to U.S.P. Thyrotropin Reference Standard reacted to a titer of 1:3000 with this hormone and to a titer of 1:1500 with "Thytropar." Neither of these antisera cross-reacted with bovine or human ACTH,

with bovine STH or posterior pituitary extract, or with ovine LH or FSH. This specificity was confirmed by the complement-fixation test. Antisera diluted 1:50 or greater fixed 3 M.H.D. of complement when tested with 0.1 unit/ml. of thyrotropin. Complement was not fixed with undiluted serum when bovine or human ACTH was used as antigen. The Ouchterlony plate showed a single line between the antiserum and bovine TSH (U.S.P. Standard) and also a single line between the antiserum and bovine STH. No precipitation lines occurred with this technique when human or bovine ACTH, ovine LH, or ovine FSH were used as antigens.

In the hemagglutination-inhibition test, rabbit antiserum diluted 1:100 was absorbed with varying concentrations of bovine TSH. Absorption of an equal volume of diluted serum with 0.5 milliunit/ml. of TSH removed all detectable thyrotropin antibody, and absorption with 0.1 milliunit/ml. significantly reduced the titer of the antiserum.

The uptake of radioactive iodine by the thyroids of mice given bovine TSH was suppressed by prior administration of a single dose of the antiserum but was not altered by the administration of normal rabbit serum (Fig. 1). The anti-hormonal activity of the antiserum was substantiated by the observation that mice injected with the supernatant fluid from a thyrotropin-antiserum mixture had less thyroidal radioactive iodine uptake than mice treated with a mixture of TSH and normal rabbit serum (Fig. 1). The differences between antiserum-treated and control groups in both assays were significant ($P<0.001$) by the method of analysis of variance.

COMMENTS

In this study, bovine thyrotropin has been shown to be antigenic in the rabbit. The antisera obtained were tested for immunologic specificity by hemagglutination and complement-fixation tests and by the Ouchterlony technique using various bovine, ovine, and porcine pituitary hormones as antigens. A cross-reaction was obtained only with bovine STH using the Ouchterlony technique. This STH preparation was known to have thyrotropic activity. Failure of the preparation to react with antiserum in the hemagglutination test may be due to the fact that the amount of TSH contamination was insufficient to sensitize the tanned cells.

The antiserum also inactivated the biologic effects of bovine TSH. On injection of antiserum into mice, there was significant inhibition of thyrotropic effect. In addition, incubation of the antiserum and TSH prior to administration removed most of the thyrotropic activity. Thus, the antiserum appears to be both serologically and biologically specific for thyrotropin.

During the course of these studies, a preliminary report appeared describing the preparation of rabbit thyrotropin antiserum capable of neutralizing the biologic effects of thyrotropin by prior inactivation *in vitro* (7). The results presented here are in agreement with these observations; further, the inactivation of thyrotropic activity *in vivo* has been demonstrated.

Hemagglutination-inhibition assay permits the detection of as little as 0.1 milliunit/ml. of TSH. Attempts are being made to develop this assay for the measurement of TSH in human sera. Studies are also in progress with the specific thyrotropin antiserum to determine the site of hormone production using the Coons' fluorescent technique.

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METABOLISM OF ESTROGENS IN THE PREGNANT MARE[1]

ABSTRACT

Administration of dihydroequilenin-17β-4-C^{14} to a pregnant mare resulted in the isolation from the urine of radioactive equilenin (and dihydroequilenin-17β). Estrone and equilin were isolated devoid of radioactivity.

The biosynthetic origins of the ring-B unsaturated estrogens, equilin and equilenin have been the subject of considerable speculation. Girard and associates (1,2) first isolated these compounds, in addition to estrone, from the urine of pregnant mares, and observed that their proportions increased during the latter months of pregnancy, at a time when the total estrogen (estrone) content of the urine diminished (2,3). Heard and associates (4) reported that estrone-C^{14} administered to a pregnant mare was not transformed to equilin nor equilenin (5). We have explored in the mare the possibility of the transformation of a ring-B unsaturated estrogen into estrone, by the administration of dihydroequilenin-17β-4-C^{14}; the only radioactive substances found in the urinary estrogens were equilenin and dihydroequilenin-17β.

METHODS

The general methods used for the preparation of the phenolic fraction of the urinary steroids, the Girard separation of the ketonic fraction, the determination of melting points, radioactivity, etc., are those described in a previous study (6).

Chromatography. The phenolic ketone fractions were chromatographed on Celite (Johns-Manville No. 545), impregnated with sodium hydroxide, modified for the ketonic estrogens (Savard and Burdulis, in preparation) from the methods of Haenni, Carol and Banes (7).

Measurement of radioactivity. All samples were plated in duplicate on aluminum dishes and were counted at infinite thinness in a flow-gas counter operating at 30% efficiency; a total of 4000 counts was accumulated for each sample. Specific activity is expressed as counts per minute per milligram (c.p.m. per mg.).

EXPERIMENTAL AND RESULTS

A hybrid mare, in the 10th month of pregnancy, was given dihydroequilenin-17β-4-C^{14} prepared from 6-dehydroestradiol-17β-diacetate-4-C^{14}(8); 22.0 mg. (approximately 24 μc.) of the chromatographically homogeneous steroid, dissolved in ethanol and physiological saline, were administered intrajugularly in a single injection. Urines were collected for the subsequent 3 days. An accidental loss of a portion of the first day's collection occurred.

The urines were treated as previously described (6) to yield a lipide extract containing 1.02×10^6 c.p.m. Most of this radioactivity was found in the phenolic fraction, from

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which the volatile phenols were removed by steam distillation prior to separation with Girard reagent. The resulting non-ketonic phenolic fraction contained 0.28×10^6 c.p.m. and the ketonic phenol fraction contained 0.52×10^6 c.p.m. and 98 mg. of estrogen (determined colorimetrically).

Non-Ketonic Phenols. A portion of this fraction (approximately 15%) was dissolved in benzene and transferred to a column of Celite (21 g.) impregnated with 0.4N sodium hydroxide (20 ml.). The column was developed with benzene and a total of 131 fractions (15 ml. each) was collected; dihydroequilenin-17β (characterized by its ultraviolet absorption spectrum (9)) was eluted in a zone comprising fractions 55–100. The amount of material (by ultraviolet absorption in methanol) and radioactivity in each tube revealed radiochemical homogeneity through this zone (10) at approximately 6,500 c.p.m. per mg.; dihydroequilenin-17β was considered to be radiochemically pure.

Ketonic Phenols. This fraction was chromatographed in portions on Celite impregnated with 1.0N sodium hydroxide and developed with benzene (7). Sixty 15-ml. fractions were collected; alternate tubes were examined to locate, characterize and measure the estrogens, the remaining tubes for radioactivity.

Equilenin. The material contained in the zone corresponding to equilenin (tubes 18–44) showed a distinct correlation between weight (by ultraviolet absorption spectra (9)) and radioactivity as evidence of radiochemical homogeneity (10). Material in this zone, from the various chromatograms, was combined, chromatographed on silica gel (11) and recrystallized several times from methanol to yield 11.4 mg. of crystalline equilenin, melting at 252–258° C (corrected), and showing the characteristic ultraviolet absorption spectrum (in methanol) and a specific activity of 6,360 c.p.m. per mg. Further crystallization did not change the specific activity, nor did formation of the picrate (1) and regeneration of the free steroid. Equilenin was therefore presumed to be radiochemically pure.

Estrone and Equilin. Examination of the weight of estrogen and radioactivity per tube in tubes 4–11 from the same chromatogram revealed little or no correlation between the two parameters and strongly suggests that the radioactivity present is a contaminant. All material in this zone from the various chromatograms was combined to yield a total of 70.5 mg. of estrogen (determined colorimetrically), consisting of estrone and equilin in a ratio of approximately 5 to 3; the radioactivity was 95 c.p.m. per mg. That most of this radioactivity was due to contamination was illustrated by the following: One-fifth of the total mixture was rechromatographed on a Celite—1.0N sodium hydroxide column, developed with ligroin:benzene (1:1) (Savard and Burdulis, in preparation). In this case, both estrogens separated into two distinct zones (peaks separated by approximately 15 tubes); again radioactivity was spread in an uncorrelated fashion in the earlier tubes; the highest level of radioactivity in the estrone zone was 5 c.p.m. per mg. (tube 12). The remaining unchromatographed estrogen mixture was subjected to an 8-transfer countercurrent distribution between 1.0N sodium hydroxide and toluene (12). Almost all the radioactivity was found in the neutral (toluene) phase; the neutral radioactive material appeared to be equilenin acetate by its ultraviolet absorption spectrum (13).

DISCUSSION

The data described in the foregoing reveal that following the administration of dihydroequilenin-17β-4-C^{14} to a pregnant mare, only two radioactive steroids, namely equilenin and dihydroequilenin-17β, were isolated from the urine. Their specific activities, approximately 6,400 c.p.m. per mg., indicate a ready biological interchange between the naphthalenic estrogens with 17-ketone and 17β-hydroxyl functions, in parallel with

the interchangeability of estrone and estradiol-17β (14,15). The radioactivity in dihydroequilenin-17β confirms its earlier isolation from the phenolic non-ketonic fraction of mares' urine (16–18).

The simultaneous isolation of urinary estrone and equilin, both devoid of significant radioactivity, following the administration of dihydroequilenin-17β-C^{14} indicates without a doubt, that little if any of the administered steroid was converted by the pregnant mare into urinary estrone or equilin.

The enigma of the biochemical significance of the ring-B unsaturated estrogens of the pregnant mare, thus remains. Neither equilin nor equilenin are formed from exogenously administered estrone (5), nor is estrone (or equilin) formed from equilenin, as this study shows. Thus neither the process of dehydrogenation of estrone (2) nor the hydrogenation of equilenin (19) appear to occur to a significant degree in the pregnant mare (at least from exogenously administered steroid). Both estrone and the ring-B unsaturated estrogens, equilin and equilenin, are formed in the mare from radioactive acetate, though less amounts of isotope appear in the latter two in comparison to the incorporation in estrone (4, 6). However, in the transformation of testosterone-C^{14} to radioactive urinary estrone in the mare, no radioactive equilin nor equilenin were formed (20).

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PROLACTIN AND THE PHOTOPERIODIC TESTICULAR RESPONSE IN WHITE-CROWNED SPARROWS

ABSTRACT

In three separate experiments it was not possible to prevent the photoperiodic testicular response in the White-crowned Sparrow, *Zonotrichia leucophrys gambelii*, by injection of prolactin. Since these results fail to confirm earlier experiments, current hypotheses concerning the role of prolactin in refractoriness to photoperiodic stimulation must be re-examined.

It is well known that artificial or natural photoperiodic stimulation of gonadal cycles in many temperate-zone species of birds is followed by a refractory period in which photoperiodic stimulation is ineffective (1, 2). Among the suggested mechanisms of refractoriness is an inhibitory effect of prolactin on some element of the response system (1, 2, 3). The primary basis for such a suggestion is the experiment by Bailey (4) in which daily subcutaneous injections of 15 I.U. of prolactin completely prevented the photoperiodic testicular response in *Zonotrichia leucophrys pugetensis*. This hypothesis appears to have been strengthened somewhat by the observation that injections of prolactin inhibit spermatogenesis in the domestic pigeon (5), and in the domestic fowl (6), and further that single intramuscular injections of 20–25 I.U. cause inhibition of spermatogenesis and tubular metamorphosis in *Passer domesticus*, *Chloris chloris*, and *Fringilla coelebs* (7). The observed tubular metamorphosis is associated temporally with the development of refractoriness (7) and may have a functional relationship to this phenomenon.

To test the hypothesis that prolacting is involved in the development of refractoriness, we have repeated the experiments of Bailey (4) with a different subspecies of White-crowned Sparrow, *Zonotrichia leucophrys gambelii*.

EXPERIMENTAL BIRDS AND METHODS

The experimental birds were captured from a wintering population in the Snake River Canyon, Whitman County, Washington, and maintained in accordance with the standard procedures of this laboratory (8). During the experiments the birds were placed in individual cages and housed indoors in light-proof rooms at 22° C. The rooms were illuminated with incandescent lamps for twenty hours each day, the intensity at the floors of the cages being about 400 lux. The details of the experiments are recorded in Table 1. In the first two experiments the hormone (Luteotrophin Squibb) was dissolved in distilled water with 0.5% phenol preservative to a concentration of 200 I.U. per ml. The control solution used (Group a) was 2% bovine serum albumin. In the third experiment a different lot of Luteotrophin Squibb was made up to concentrations of 150 I.U. per ml. (Group b) and 750 I.U. per ml. (Group c) in isotonic saline, the control solution (Group a) being isotonic saline alone. Subcutaneous injections were administered daily at a different site as in Bailey's experiments (personal communication). At the close of each experiment birds were autopsied; the testes were processed and testicular growth rates calculated according to the usual procedures of our laboratory (8). The testicular growth-rate constants, k, as shown in the table, represent the slopes of the regression lines for plots of logarithms of testicular weights versus time in days. These,

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the interchangeability of estrone and estradiol-17β (14,15). The radioactivity in dihydroequilenin-17β confirms its earlier isolation from the phenolic non-ketonic fraction of mares' urine (16–18).

The simultaneous isolation of urinary estrone and equilin, both devoid of significant radioactivity, following the administration of dihydroequilenin-17β-C^{14} indicates without a doubt, that little if any of the administered steroid was converted by the pregnant mare into urinary estrone or equilin.

The enigma of the biochemical significance of the ring-B unsaturated estrogens of the pregnant mare, thus remains. Neither equilin nor equilenin are formed from exogenously administered estrone (5), nor is estrone (or equilin) formed from equilenin, as this study shows. Thus neither the process of dehydrogenation of estrone (2) nor the hydrogenation of equilenin (19) appear to occur to a significant degree in the pregnant mare (at least from exogenously administered steroid). Both estrone and the ring-B unsaturated estrogens, equilin and equilenin, are formed in the mare from radioactive acetate, though less amounts of isotope appear in the latter two in comparison to the incorporation in estrone (4, 6). However, in the transformation of testosterone-C^{14} to radioactive urinary estrone in the mare, no radioactive equilin nor equilenin were formed (20).

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PROLACTIN AND THE PHOTOPERIODIC TESTICULAR RESPONSE IN WHITE-CROWNED SPARROWS

ABSTRACT

In three separate experiments it was not possible to prevent the photoperiodic testicular response in the White-crowned Sparrow, *Zonotrichia leucophrys gambelii*, by injection of prolactin. Since these results fail to confirm earlier experiments, current hypotheses concerning the role of prolactin in refractoriness to photoperiodic stimulation must be re-examined.

It is well known that artificial or natural photoperiodic stimulation of gonadal cycles in many temperate-zone species of birds is followed by a refractory period in which photoperiodic stimulation is ineffective (1, 2). Among the suggested mechanisms of refractoriness is an inhibitory effect of prolactin on some element of the response system (1, 2, 3). The primary basis for such a suggestion is the experiment by Bailey (4) in which daily subcutaneous injections of 15 I.U. of prolactin completely prevented the photoperiodic testicular response in *Zonotrichia leucophrys pugetensis*. This hypothesis appears to have been strengthened somewhat by the observation that injections of prolactin inhibit spermatogenesis in the domestic pigeon (5), and in the domestic fowl (6), and further that single intramuscular injections of 20–25 I.U. cause inhibition of spermatogenesis and tubular metamorphosis in *Passer domesticus*, *Chloris chloris*, and *Fringilla coelebs* (7). The observed tubular metamorphosis is associated temporally with the development of refractoriness (7) and may have a functional relationship to this phenomenon.

To test the hypothesis that prolacting is involved in the development of refractoriness, we have repeated the experiments of Bailey (4) with a different subspecies of White-crowned Sparrow, *Zonotrichia leucophrys gambelii*.

EXPERIMENTAL BIRDS AND METHODS

The experimental birds were captured from a wintering population in the Snake River Canyon, Whitman County, Washington, and maintained in accordance with the standard procedures of this laboratory (8). During the experiments the birds were placed in individual cages and housed indoors in light-proof rooms at 22° C. The rooms were illuminated with incandescent lamps for twenty hours each day, the intensity at the floors of the cages being about 400 lux. The details of the experiments are recorded in Table 1. In the first two experiments the hormone (Luteotrophin Squibb) was dissolved in distilled water with 0.5% phenol preservative to a concentration of 200 I.U. per ml. The control solution used (Group a) was 2% bovine serum albumin. In the third experiment a different lot of Luteotrophin Squibb was made up to concentrations of 150 I.U. per ml. (Group b) and 750 I.U. per ml. (Group c) in isotonic saline, the control solution (Group a) being isotonic saline alone. Subcutaneous injections were administered daily at a different site as in Bailey's experiments (personal communication). At the close of each experiment birds were autopsied; the testes were processed and testicular growth rates calculated according to the usual procedures of our laboratory (8). The testicular growth-rate constants, k, as shown in the table, represent the slopes of the regression lines for plots of logarithms of testicular weights versus time in days. These,

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TABLE 1. THE EFFECT OF PROLACTIN ON THE PHOTOPERIODIC TESTICULAR RESPONSE IN THE WHITE-CROWNED SPARROW, *Zonotrichia leucophrys gambelii*

Experiment	Initial date	Duration, days	Group	20-hour birds				Short-day control[1]	
				No. of birds	Daily injection[2]	Testicular wt. mean, mg.	k[3], days^{-1}	No. of birds	Testicular wt. mean, mg.
I	19 Feb. 1958	20	a	2	albumin	85.4	0.095 ± 0.043	5	1.11
		20	b	3	15 I.U. prolactin	154	0.113 ± 0.018	6	1.01
II	3 Dec. 1958	20	a	2	albumin	25.5	0.078 ± 0.017	7	0.64
		20	b	6	15 I.U. prolactin	26.0	0.079 ± 0.017	6	0.65
III	29 Dec. 1959	20	a	4	saline	43.7	0.095 ± 0.011	4	0.62
		20	b	9	15 I.U. prolactin	50.4	0.100 ± 0.005	10	0.68
		20	c	9	75 I.U. prolactin	57.6	0.104 ± 0.010	10	0.59
	1 Jan. 1960	19	d	5	none	54.0	0.101 ± 0.002	10	0.79

[1] Held on non-stimulatory photoperiods (9 hrs.).
[2] Injections were begun 1–4 days before increasing the daily photoperiod from 8 to 20 hours.
[3] Logarithmic testicular growth rate constant (8) and its 95% fiducial limits.

with their 95% fiducial limits, provide a basis for comparison of the photoperiodic testicular responses.

The prolactin used in Experiment III was assayed by the method of Reece and Turner (9); the results confirm the assay of the manufacturer within the error of the method. Hence it is certain that the dosages in Group b were as great as those used by Bailey whereas those in Group c were substantially greater.

RESULTS AND DISCUSSION

As may be seen from the fiducial limits of k in Table 1 there is clearly no reason to suspect that prolactin has reduced the photoperiodic responses of the treated birds. The differences among experiments with respect to testicular growth rate may be correlated with season rather than with experimental conditions (10).

So far as we are aware, our experiments and those of Bailey represent the only attempts to prevent photoperiodic testicular responses with prolactin. Since we have duplicated his procedures as completely as possible, the strikingly incongruous results are difficult to explain. A subspecific difference is, of course, possible although this seems unlikely. Similarly our use of 20-hour photoperiods, rather than continuous light, for photostimulation appears to be an unlikely explanation (8). Possibly the method of preparation of Squibb Luteotrophin has been altered since Bailey's investigations so that a trace of another active compound has been lost from the preparation, or so that some active group may no longer be present. Whatever the explanation for the difference in results may be, hypotheses relating prolactin to photoperiodic refractoriness in wild birds must be treated with caution until further experimental data are available.

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Acknowledgments

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COMPARATIVE EFFECTIVENESS OF PROGESTERONE AND 4-PREGNEN-20α-OL-3-ONE IN THE DEVELOPMENT OF DECIDUOMATA

ABSTRACT

The comparative effectiveness of subcutaneous injections of progesterone and 4-pregnen-20α-ol-3-one in oil to elicit the decidual reaction in ovariectomized rats was determined. While progesterone was shown to have activity, 4-pregnen-20α-ol-3-one was ineffective in doses up to 3 mg. per day.

Zander, *et al.* (1) reported that 4-pregnen-20α-ol-3-one and 4-pregnen-20β-ol-3-one, two naturally occurring metabolites of progesterone, possess biological activity in progestational assays. The former compound was judged to have activity 1/2 to 1/3 that of progesterone in the Clauberg test and 1/2.5 in the Hooker-Forbes assay, while the latter steroid was twice as potent in the Hooker-Forbes test but only 1/5 to 1/10 as active in the Clauberg test. The demonstration that both progesterone and 4-pregnen-20α-ol-3-one are endogenous to the rat during the latter third of pregnancy (2) indicated the need of assessing the relative potency of the 20α-hydroxy derivative in the rat. This was accomplished by comparing the abilities of the two steroids to support decidual development in the ovariectomized rat.

METHODS

The procedures used were essentially those described by Hisaw and Velardo (3). In brief, pseudopregnancy was induced in the rats by electrical stimulation of the uterine cervix. The animals were castrated on the fourth day of pseudopregnancy (fifth day after electrical stimulation), and the right horn of the uterus was traumatized throughout its entire length by scratching the antimesometrial surface of the endometrium. Daily doses of the two steroids in 0.1 ml. of sesame oil were injected subcutaneously for three days. Seventy-two hours after the initial injections the rats were killed, and the uteri were weighed on a pharmaceutical torsion balance.

RESULTS AND DISCUSSION

The ability of progesterone to support decidual development is clearly seen (Table 1), however, the maximum dose was not sufficient to elicit a reaction comparable to that

Table 1. Action of progesterone and 4-pregnen-20α-ol-3-one in eliciting decidual development

No. of rats	Mg. steroid daily dose	Traumatized horn, in mg.	Control horn, in mg.	% increase* over control horn
6	0.0	165±11	129±11	29±8
4	0.5, Progest.	321±71	143±11	128±68
4	1.0, Progest.	753±71	145±18	429±107
4	1.5, Progest.	971±109	143±17	588±105
3	0.0**	1683±169	138±15	1098±118
12	1.0, Pregnen.	133±15	123±18	9±7
10	1.5, Pregnen.	157±25	136±20	19±10
10	3.0, Pregnen.#	153±23	130±11	18±15

* $\frac{\text{(Wt. traum. horn)} - \text{(Wt. control horn)}}{\text{(Wt. control horn)}} \times 100.$

** Positive control rats possessing intact ovaries.

3.0 mg. 4-Pregnen-20α-ol-3-one per 0.1 ml. sesame oil constitutes a supersaturated solution. The actual dose administered was somewhat less than indicated.

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found in normal pseudopregnant rats (cf. 3). In contrast to the effectiveness of progesterone, it is apparent that 4-pregnen-20α-ol-3-one[1] was ineffective in this test. While these results do not necessarily establish that 4-pregnen-20α-ol-3-one is without progestational activity in the rat, since the administration of larger doses was prohibited by solubility limitations, they do indicate that 4-pregnen-20α-ol-3-one has less than 1/6th the progestational potency of progesterone in supporting the decidual reaction in the rat.

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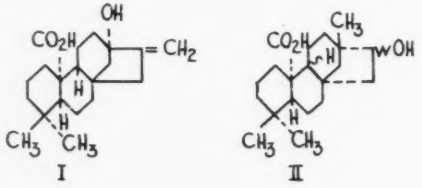
[1] Generously supplied by The Upjohn Company, Kalamazoo, Mich.

ANTI-ANDROGENIC ACTIVITY OF DIHYDROISOSTEVIOL[1]

ABSTRACT

Dihydroisosteviol, when inuncted to the chick's comb, inhibited the action of injected testosterone. At the doses studied, dihydroisosteviol did not inhibit the action of testosterone on the seminal vesicles, prostate, and levator ani muscle of the castrated rat.

Anti-androgenic activity has been reported for various nonsteroidal compounds including a derivative of perhydrophenanthrene (2-acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene: Ro 2-7239) in the rat (1) and the chick (2), methylcholanthene in the chick (3, 4), and benzpyrene in the chick (4). We are now reporting studies on the anti-androgenic activity of two additional nonsteroid compounds, viz., steviol (I) and dihydroisosteviol (II), which are diterpenoid acids derived from the intensely sweet glycoside of the Paraguayan herb, Stevia Rebaudiana (5, 6, 7). While the structures shown for I and II are the most likely ones, the position of the carboxyl group and the sterochemistry of the fusion between rings B and C have not yet been definitely established.[2]



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[1] Supported in part by U. S. Public Health Service Grants No. CY-2193, CRT -5001 and RG-6019 of the National Institutes of Health.
[2] Private communication from Dr. Erich Mosettig.

TABLE 1. THE ANTI-ANDROGENIC ACTIVITY OF STEVIOL AND DIHYDROISOSTEVIOL IN A CHICK COMB TEST

Experiment No.	Compound inuncted (total dose, mg.)	Total dose of testosterone enanthate, mg.	No. of chicks	Mean comb ratio* ± S.E.
A	0	0	25	0.37 ± 0.018
	0	0.5	25	0.92 ± 0.039
	Norethisterone (0.25)	0.5	25	0.64 ± 0.028
	Norethisterone (1.0)	0.5	25	0.50 ± 0.019
	Steviol (0.5)	0.5	10	0.88 ± 0.071
	Steviol (2.0)	0.5	10	0.84 ± 0.050
B	0	0	20	0.33 ± 0.012
	0	0.5	20	0.86 ± 0.039
	Norethisterone (0.25)	0.5	20	0.72 ± 0.028
	Norethisterone (1.0)	0.5	19	0.59 ± 0.024
	Steviol (2.0)	0.5	10	0.74 ± 0.940
C	0	0	20	0.47 ± 0.015
	0	0.5	20	1.09 ± 0.074
	Norethisterone (0.25)	0.5	20	0.97 ± 0.034
	Norethisterone (1.0)	0.5	20	0.80 ± 0.048
	Dihydroisosteviol (0.5)	0.5	10	0.98 ± 0.029
	Dihydroisosteviol (3.0)	0.5	10	0.77 ± 0.072
	Steviol (0.5)	0.5	10	1.03 ± 0.074
	Steviol (3.0)	0.5	9	0.92 ± 0.055
D	0	0	15	0.35 ± 0.022
	0	0.5	14	0.90 ± 0.051
	Ro 2-7239 (0.5)	0.5	14	0.73 ± 0.045
	Ro 2-7239 (1.5)	0.5	14	0.46 ± 0.016
	Dihydroisosteviol (1.5)	0.5	14	0.77 ± 0.049
	Dihydroisosteviol (3.0)	0.5	14	0.70 ± 0.041

* Comb ratio = mg. of comb per gm. of body weight.

EXPERIMENTAL

The anti-androgenicity of steviol and dihydroisosteviol in the chick was determined by a previously described method (2) using the one to two-day old white leghorn male chick stimulated by testosterone enanthate. The androgen (0.5 mg.) was administered once in 0.5 ml. of sesame oil on the first day of the assay, while the compounds were inunected once daily for 7 days in 0.05 ml. of a sesame oil solution. Two control groups were employed, one in which only oil was inunected to the comb, and a second group which received testosterone enanthate by injection and the vehicle only inunected once daily for the 7-day period. Norethisterone (17α-ethynyl-19-nortestosterone) and Ro 2-7239 served as standards.

After seven days the combs were removed, blotted and weighed on a torsion balance to the nearest 0.5 milligrams. The results are expressed in terms of comb ratio, which is defined as milligrams of comb per gram of body weight.

Dihydroisosteviol was also tested for possible anti-androgenic activity in the castrated rat stimulated with testosterone. For this test 28-day old Charles River male rats were castrated and subcutaneous injections started on the day of surgery. Testosterone was injected once daily in 0.5 ml. of an aqueous suspension[3] for 7 days and at another site dihydroisosteviol was similarly injected. This latter compound was suspended in the same aqueous medium that was used for the testosterone and injected in 0.5 ml. per day for 7 days. On the 8th day

TABLE 2. INFLUENCE OF DIHYDROISOSTEVIOL ON THE CASTRATED RAT IN PRESENCE AND ABSENCE OF INJECTED TESTOSTERONE

Experiment No.	Total dose of Dihydroisosteviol, mg.	Total dose of testosterone, mg.	No. of rats	Mean body wt. gm.	Mean tissue ratio* ± S.E.		
					Seminal vesicles	Prostate	Levator ani
E	0	0	9	80	0.09±0.007	0.14±0.015	0.22±0.024
	0	2.4	9	91	1.36±0.103	1.40±0.125	0.56±0.026
	5	0	5	90	0.11±0.010	0.20±0.939	0.23±0.043
	5	2.4	5	88	1.29±0.072	1.44±0.050	0.47±0.057
F	0	0	5	93	0.07±0.005	0.13±0.018	0.23±0.017
	0	2.4	9	90	1.10±0.079	1.31±0.038	0.69±0.053
	20	0	5	94	0.80±0.006	0.14±0.013	0.25±0.006
	20	2.4	5	92	1.04±0.142	1.48±0.119	0.67±0.047

Ratio = mg. of tissue per gm. of body weight.

the rats were sacrificed and the weights of the seminal vesicles, prostate, and levator ani determined together with the body weight. The results are expressed as mg. of tissue per gm. of body weight.

The results of four chick anti-androgen assays are summarized in Table 1. In three trials with steviol at the 2 to 3 mg. level a tendency toward anti-androgenic activity was observed. Two trials at the 0.5 mg. dose level of steviol were negative. Dihydroisosteviol failed to produce a statistically significant inhibition at the 0.5 and 1.5 mg. dose levels but significant interference with the action of testosterone was demonstrated twice with 3.0 mg. (Experiments C ($P = <0.02$) and D ($P = <0.01$)). At the 3.0 mg. levels the inhibition was 51% and 63%.

Dihydroisosteviol neither stimulated the seminal vesicles, prostate, and levator ani nor was this compound effective in inhibiting the action of testosterone at total dose levels of 5 and 20 mg. (Table 2).

Acknowledgments

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[3] This vehicle consists of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethylcellulose (0.5%) and benzyl alcohol (0.9%).

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SOME CHANGES OF APPETITE IN ALLOXAN DIABETIC RATS[1]

ABSTRACT

An experiment was carried out during which alloxan diabetic and normal rats were offered water and 1% solutions of glucose, NaCl, KCl, Na_2HPO_4 and NaH_2PO_4 as drinking fluids. The results showed considerable differences in appetite between alloxan diabetic and normal rats. Alloxan diabetic rats exhibited a preference for the glucose solution and tap water, whereas normal animals preferred solutions of NaH_2PO_4 and NaCl. Alloxan diabetic rats showed a marked appetite for Na_2HPO_4.

Animals are known to be able to adapt to some degree their food and fluid intake to the requirements of their organism. This ability manifests itself in quantitative and qualitative changes of appetite in the course of different pathological conditions, a fact shown by numerous authors like Richter and Eckert (1), Harris and collaborators (2), Clark and Clausen (3), as well as by Scott and coworkers (4). Andik (5), and Vartiainen and Paasonen (6) have reported data on the changes of appetite in alloxan diabetic rats.

Prompted by these works, we have tried to determine if alloxan diabetic rats, as compared with normal rats show changes in their selection of certain solutions when simultaneously fed Coward's (7) diet *ad libitum*.

ANIMALS AND METHODS

Twelve male albino rats with a body weight of 240–270 gm. were used for the experiment. The experimental and control group consisted of 6 rats each. Alloxantetrahydrate (Merck) 60 mg. per kg. body weight was injected intravenously into the experimental rats. Fasting blood sugar level in the diabetic rats ranged from 230 to 290 mg. per 100 cc.

Beginning from the 10th day after injection of alloxan, both normal and alloxan diabetic rats were offered six different solutions to drink as follows: 1. tap water, 2. 1% glucose, 3. 1% NaCl, 4. 1% KCl, 5. 1% Na_2HPO_4 and 6. 1% NaH_2PO_4. All the solutions were prepared with tap water except Na_2HPO_4 which is more soluble in distilled water. These solutions varied in molarity and pH. The pH values for tap water and NaCl and KCl solutions ranged from 7.5 to 7.7, that of Na_2HPO_4 was 8.8, and that of NaH_2PO_4 5.9.

Accordingly, each group of animals was offered a set of six bottles, identical in appearance, each bottle containing one of the six drinking fluids. During the experiment which lasted for nine days, each set of bottles was interchanged daily from one cage to another, and apart from that the relative positions of bottles were also changed every day. The bottles were weighed daily and the fluid intake of each group as a whole was calculated from the consumption. An individual animal having such a variety of drinks at its disposal would consume quantities of some drinks too small to be weighed.

RESULTS AND DISCUSSION

The results are shown in Figure 1. Columns A show quantitative difference in the total fluid intake between diabetic and normal rats. While the amount of fluid drank by

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[1] Reported in abstract form at the III Congress of the International Diabetes Federation. Düsseldorf, July 21–25, 1958.

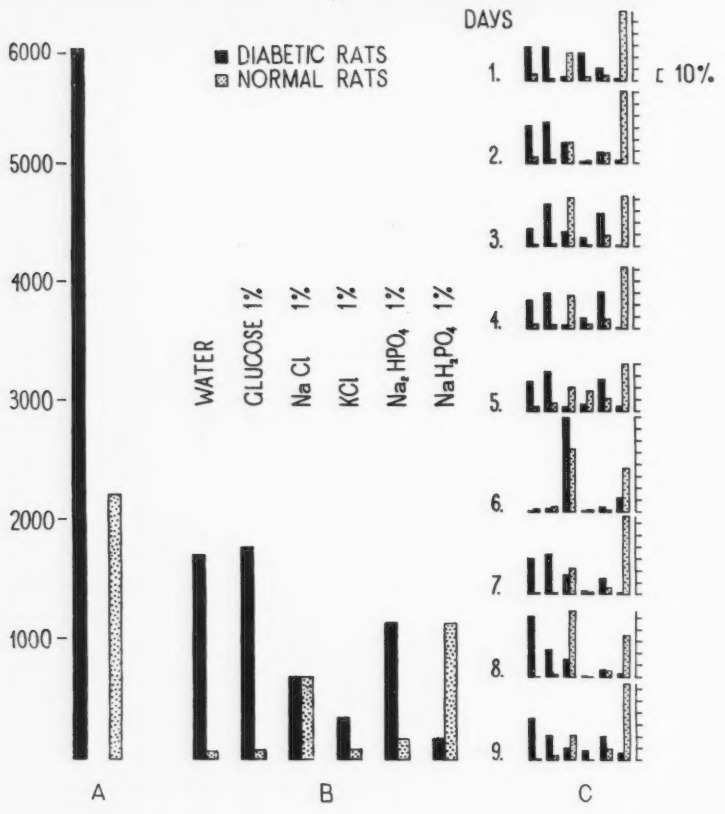

FIG. 1.

diabetic animals in the 9-day experimental period was 6029 gm., normal animals drank in the same period 2242 gm. total fluid.

Columns B show qualitative differences in the fluid intake. Self selection in the experimental group differed essentially from that in the normal group. In diabetic animals compared with normal ones there was a greater water intake than would be expected considering the diabetic polydipsia presented under A. There is also a very pronounced difference between normal and diabetic animals as regards their appetite for glucose solution. Diabetic rats manifested a striking appetite for that solution, while the normal ones neglected it almost entirely. The intake of NaCl solution as expressed in absolute values is the same in both groups but represents a small fraction of total fluid intake in diabetic rats. Interest in KCl solution was small in both groups. A marked difference is evident in intakes of Na_2HPO_4 and NaH_2PO_4 solutions. Alloxan diabetic rats preferred the Na_2HPO_4 solution, while normal animals exhibited preference for the NaH_2PO_4 solution.

Columns C demonstrate intake of different fluids expressed as percent of total fluid intake per each day of the experiment.

We have performed the statistical analysis using the Wald-Wolfowitz (8) runs test, which showed a significant difference in the quantities of water, 1% glucose, 1% NaCl and 1% NaH_2PO_4 expressed in terms of total fluid intake per day (Fig. 1 C) between diabetic and normal rats (H_0 can be rejected at the 0.05 level of significance). The consumption of other fluids expressed in the same way does not show significant differences between the two groups.

It is very interesting to note that diabetic rats have a great appetite for glucose solution. Normal rats given a variety of drinks will also select glucose, but they prefer only the more concentrated solutions. The mechanism whereby this change of appetite is induced is unknown. However, the fact that diabetic rats select glucose solution is not surprising if we bear in mind that they are actually in a condition resembling privation of carbohydrates. Metabolism in these animals is so profoundly disturbed that utilization of glucose is very low and loss of glucose in the urine tremendous. In an attempt to compensate for the faulty utilization of sugar diabetic animals produce it by gluconeogenesis. Could not we suppose that another mechanism comes into action which by change of appetite tends to supply the body with necessary carbohydrates? We do not want to imply that this change of appetite will improve the condition of diabetic animals, though it should be borne in mind that ketosis may be decreased by large amounts of carbohydrates as shown by Mirsky and coworkers (9). In diabetic patients following ingestion of glucose the generally low concentration of bicarbonate is increased in serum. Van Eck (10) showed that the condition of diabetic patients can be improved by administration of carbohydrates in larger amounts than has been advocated in the past. He reported that diabetic patients tolerate rather well the increased quantities of carbohydrates in the diet if the amount of fat is decreased. This does not increase their need for insulin. As regards the increased appetite of alloxan diabetic rats for Na_2HPO_4, it is probably related to a fall in the alkali reserve.

The explanation that the changed behavior of alloxan diabetic rats concerning self-selection of drinking fluids is due to changed physiological needs would of course be quite teleological. However, as the experiments presented here indicate, the reaction of these animals is rather adequate and tends to maintain homeostasis. The mechanism of these psychophysiological phenomena remains to be elucidated.

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THE ENDOCRINE SOCIETY

1961 Annual Meeting

The next annual meeting of The Endocrine Society will be held in the Hotel Biltmore, New York, N. Y. June 22–24, 1961.

TWELFTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

October 24–28, 1960

PROGRAM

Monday Morning, October 24

8:00– 9:00 Registration: Exhibit Gallery of the Wisconsin Center Building

9:00–12:30 Hypothalamic and Pituitary Disease. (Chairman: Dr. Charles W. Lloyd)

Regulation of Endocrine Function and the Nature of Pituitary Hormones —Dr. Roland K. Meyer and Dr. William H. McShan

Hyperactivity of the Pituitary—Dr. Raymond V. Randall

The Diagnosis of Hypothalamic and Pituitary Failure—Dr. Charles W. Lloyd

Treatment of Pituitary Failure—Dr. Edgar S. Gordon

Panel Discussion of Cases—Drs. Lloyd, Gordon, McShan, Meyer and Randall

Monday Afternoon

2:00– 5:30 Calcium and Bone Metabolism. (Chairman: Dr. F. Raymond Keating, Jr.)

Some Basic Concepts of the Anatomy and Physiology of Bone Tissues — Dr. John E. Howard

Changing Concepts of Osteoporosis—Dr. G. Donald Whedon

Laboratory Evaluation of Bone Calcium Metabolism and Parathyroid Metabolism—Dr. Theodore B. Schwartz

Clinical Aspects of Hyperparathyroidism—Dr. F. Raymond Keating, Jr.

Panel Discussion of Cases—Drs. Keating, Howard, Schwartz and Whedon

Tuesday Morning, October 25

9:00–12:30 Thyroid. (Chairman: Dr. Rulon W. Rawson)

Hormone Synthesis and Hormone Transport—Dr. Joseph E. Rall

Metabolism of Thyroid Hormones—Dr. Edwin C. Albright

Panel Discussion of Cases—Drs. Rawson, Albright, Keating, Rall and Turner

Tuesday Afternoon

2:00– 5:30 Thyroid. (Chairman: Dr. Brown Dobyns)

Mechanisms of Action of the Thyroid Hormone—Dr. Henry Lardy

Genetic Factors in Simple Goiter—Dr. Judson J. Van Wyk
Panel Discussion of Cases—Drs. Dobyns, Lardy, McCullagh, Rawson and Van Wyk

Wednesday Morning, October 26

9:00–12:30 ADRENAL CORTEX. (Chairman: Dr. Peter H. Forsham)
Introduction—Dr. Peter H. Forsham
Adrenal Steroids—Dr. Judson J. Van Wyk
Central Nervous System Regulation of Adrenal Cortical Function—Dr. William F. Ganong
Addison's Disease—Dr. Peter H. Forsham
Synthetic Steroids—Dr. Grant W. Liddle
Panel Discussion: Use of Anti-inflammatory Steroids—Drs. Forsham, Ganong, Liddle and Van Wyk

Wednesday Afternoon

2:00– 5:30 ADRENAL CORTEX. (Chairman: Dr. Grant W. Liddle)
Primary Aldosteronism—Dr. Jerome W. Conn
Secondary Aldosteronism—Dr. Edgar S. Gordon
Cushing's Syndrome—Dr. Randall G. Sprague
ACTH Suppression in Diagnosis of Cushing's Syndrome—Dr. Grant W. Liddle
Virilizing Congenital Adrenal Hyperplasia—Dr. Judson J. Van Wyk
Panel Discussion: Treatment of Hyperactivity of the Adrenal Cortex—Drs. Liddle, Conn, Forsham, Hertz, Sprague and Van Wyk

Thursday Morning, October 27

9:00–12:30 TESTIS. (Chairman: Dr. Warren O. Nelson)
Embryogenesis of the Gonads and Gonadal Dysgenesis—Dr. Warren O. Nelson
Hypogonadism and Gynecomastia—Dr. E. Perry McCullagh
Cryptorchidism and Male Climacteric—Dr. John E. Howard
Sexual Precocity—Dr. David W. Smith
Male Infertility—Dr. Richard L. Landau
Panel Discussion of Cases—Drs. Nelson, Howard, Landau, McCullagh and Smith

Thursday Afternoon

2:00– 5:30 OVARY. (Chairman: Dr. Robert P. Greenblatt)
Introduction—Dr. Robert P. Greenblatt
Progesterone and Its Metabolic Effects—Dr. Richard L. Landau
Treatment of Menstrual Disorders—Dr. Robert P. Greenblatt
Treatment of Endometriosis—Dr. Robert W. Kistner
Treatment of Abortion—Dr. Charles W. Lloyd
Panel Discussion of Cases—Drs. Greenblatt, Kistner, Landau and Lloyd

Friday Morning, October 28

9:00–12:30 DIABETES. (Chairman: Dr. Randall G. Sprague)
Disturbances of Intermediary Metabolism in Diabetes—Dr. Rachmiel Levine
The Relation between Lipid Metabolism and Diabetes—Dr. Edgar S. Gordon

Early Detection of Diabetes, and Speculations on the Possible Prevention of Diabetes—Dr. Stefan S. Fajans
Insulin and Oral Agents in Treatment of Diabetes—Dr. Randall G. Sprague
Pituitary and Adrenal Influences on Carbohydrate Metabolism as Observed in Clinical Diabetes—Dr. E. Perry McCullagh
Questions and Answers by members of panel and registrants

Friday Afternoon

2:00– 5:30 Endocrinology and Oncology—Dr. Roy Hertz and Dr. Olaf H. Pearson
The Effect of Hormones on Lipid Metabolism—Dr. Howard Eder
Question and Answer Period

FACULTY

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Urology Award—The Americal Urological Association offers an annual award of $1000 (first prize of $500, second prize $300, and third prize $200) for essays on the result of some clinical or laboratory research in Urology. Competition is limited to Urologists who have been graduated not more than ten years, and to hospital internes and residents doing research work in Urology.

The first prize essay will appear on the program of the forthcoming meeting of the American Urological Association, to be held at the Hotel Biltmore, Los Angeles, California, May 22–25, 1961.

For full particulars write the Executive Secretary, William P. Didusch, 1120 North Charles Street, Baltimore, Maryland. Essays must be in his hands before December 1, 1960.

CORRECTION

In the note entitled "Effect of Adrenal Cortical Hormones on Eruption Rate of Incisor Teeth in the Rat" by L. Garren and R. O. Greep appearing in ENDOCRINOLOGY, 66: 625. 1960, the value for E.R. at Week 3 and 2.5 mg. dosage should read 3.48 instead of 2.48.